ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 37

★

STOCKHOLM 1956

KUNGL. BOKTRYCKERIET P. A. NORSTEDT & SÖNER

ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 37 FASC. 1

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Stockholm 1956 · P. A. Norstedt & Söner

17/7 1956

Redigenda curavit

PROFESSOR G. LILJESTRAND

KAROLINSKA INSTITUTET

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From the Department of Pharmacology, Faculty of Medicine, Gothenburg University.

Effect of Adrenaline, Noradrenaline, Isopropyl-Noradrenaline and Ephedrine on Tone and Lactic Acid Formation in Bovine Tracheal Muscle.

By

ELLA MOHME-LUNDHOLM.

Received 7 March 1956.

The broncholytic effect of various sympathomimetic drugs is of considerable interest in that these latter are among the principal symptomatic agents used in bronchial asthma. In an earlier investigation I found that adrenaline produced its relaxing effect on smooth muscle from respiratory organs by inducing lactic acid formation therein (MOHME-LUNDHOLM 1953). In the following it will be shown that noradrenaline, isopropyl-noradrenaline and ephedrine, too, have a similar action on smooth muscle from respiratory organs.

Method.

The experiments were performed on bovine tracheal muscle. About 15 minutes after slaughter of the animal the muscle was dissected out and placed in a thermos flask containing ice-cooled Tyrode's solution. Strips of the muscle, about 10 mm in width, were then cut and, after removal of connective tissue and mucosa, were suspended in an organ bath *ad modum* MAGNUS with the help of the fragments of cartilage at the attachment. The solution, which had a volume of 20 ml and temperature of 38° C, was bubbled with 6.5 per cent CO_2 in O_2. The changes in tone were recorded by means of an isotonic pen with a load of 40 g.

Five preparations were suspended simultaneously in the bath. After 15 minutes, carbaminoylcholine was added in a concentration of $1.25 \cdot 10^{-7}$ (2.5 γ/20 ml) in order to produce satisfactory initial tone. When the tone, after 5—10 minutes, had reached a constant level, l-adrenaline, l-noradrenaline, dl-isopropyl-noradrenaline and l-ephedrine were added to four different preparations; the fifth being used as a control for determination of the initial lactic acid content. Five minutes

after addition of the drugs, when the relaxing effect had developed, all preparations were taken for lactic acid assay. The lactic acid content was determined *ad modum* FRIEDEMANN, COTONIO and SCHAFFER (1927), with a few minor modifications (MOHME-LUNDHOLM 1953).

Results.

The lowest concentration of each drug that had a maximal relaxing effect was determined first; *i. e.*, the dose which, when doubled, did not have a greater effect but, when halved, produced a lesser effect. This concentration was $2 \cdot 10^{-6}$ (40 γ/20 ml) for l-adrenaline, $4 \cdot 10^{-6}$ (80 γ/20 ml) for l-noradrenaline, $2 \cdot 10^{-5}$ (200 γ/20 ml) for dl-isopropyl-noradrenaline, and $3.5 \cdot 10^{-4}$ (7 mg/20 ml) for l-ephedrine.

The maximal relaxing effect varied a good deal from one experiment to another, but in each particular experiment adren-

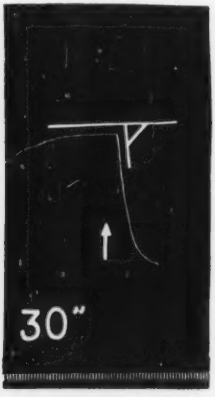

Fig. 1. Effect of adrenaline (40 γ/20 ml) on bovine tracheal muscle. The relaxation angle was 87°.

aline or isopropyl-noradrenaline had the strongest effect, that of ephedrine and noradrenaline being weaker. Since the initial tone varied considerably in the different preparations, the variation in the absolute fall of tone was substantial. However, in order to secure an approximate quantitative figure for the relaxing effect of each drug, the angle formed by the tone- and time curves before and after addition of the respective drugs was measured (figure 1). The greatest relaxation angle — 110° — was obtained, on the average, with isopropyl-noradrenaline; the

Table I.

The effect of adrenaline, noradrenaline, isopropyl-noradrenaline and ephedrine on the lactic acid content of bovine tracheal muscle.

Increase of lactic acid content from basal values, mg per cent			
l-adrenaline $2 \cdot 10^{-6}$	l-noradrenaline $4 \cdot 10^{-6}$	dl-isopropyl-noradrenaline $2 \cdot 10^{-5}$	l-ephedrine $3.5 \cdot 10^{-4}$
41.7	16.9	50.7	25.6
10.2	7.5	11.0	25.0
12.6	— 0.1	4.9	2.5
6.6	3.7	27.2	5.6
0.9	7.7	21.2	36.8
3.3	13.8	9.6	28.9
29.1	5.8	26.2	17.6
23.3	22.0	27.7	15.6
	27.7		24.0
	16.5		
	10.3		
Mean 16.0 ± 5.01	12.0 ± 2.50	22.3 ± 5.12	20.2 ± 3.68
$0.02 > P > 0.01$	$P < 0.001$	$0.01 > P > 0.001$	$P < 0.001$

smallest — 81° — with noradrenaline. The corresponding angles for adrenaline and ephedrine were 105° and 97° respectively.

Table I shows the increase in the lactic acid content above the initial levels in respect of the different drugs. Isopropyl-noradrenaline produced, on the average, both the greatest relaxing effect and the biggest elevation of the lactic acid content. Noradrenaline had the weakest effect on lactic acid formation and tone. A correlation thus seems to exist between the relaxing effect and the increase of the lactic acid formation.

By adding ammonium carbonate in a concentration of $1 \cdot 10^{-2}$ (0.2 g/20 ml) it was possible to inhibit the relaxing effect of all sympathomimetic amines used. Various substances that depress the lactic acid forming action of adrenaline, such as copper ions, sodium fluoride, monoiodoacetic acid, sodium azid, sodium arsenate, glyceraldehyde and calcium ions (MOHME-LUNDHOLM 1953 and 1956), also totally abolished the relaxing effect of noradrenaline, isopropyl-noradrenaline and ephedrine.

Discussion.

TAINTER and CAMERON (1936) reported a ratio of 1 : 5 : 385 for the doses of, respectively, l-adrenaline, dl-noradrenaline and l-ephedrine that have the same broncholytic effect on isolated guinea-pig lung. In the present investigation, where the lowest

dose having a maximal relaxing effect was determined, the corresponding ratio was found to be 1 : 2 : 350; *i. e.*, the agreement is satisfactory having regard to the fact that I used l-noradrenaline, which is twice as active as the racemic form. On the other hand the ratio of the doses of adrenaline and isopropyl-noradrenaline (1 : 10) having a maximal relaxing effect on bovine tracheal muscle is altogether inconsistent with KONZETT's (1940) report that isopropyl-noradrenaline has a ten times greater broncholytic effect than adrenaline.

Whether the discrepancies in the results are due to differences in experimental methods or to a genuine difference in the mode of reaction of smooth muscle from different sites, is uncertain. In my experiments on isolated rabbit gut, adrenaline and isopropyl-noradrenaline had the same activity ratio as that in the experiments on tracheal muscle (MOHME-LUNDHOLM 1953).

It is especially interesting to compare the effects of ephedrine on rabbit gut and bovine tracheal muscle. On the first-named preparation ephedrine in a concentration of $3.5 \cdot 10^{-4}$ had no appreciable relaxing effect, nor any lactic acid forming effect (MOHME-LUNDHOLM 1953), but on the last-named preparation both of those effects were demonstrable.

Summary.

The relaxing and the lactic acid forming effects on bovine tracheal muscle of adrenaline, noradrenaline, isopropyl-noradrenaline and ephedrine were compared.

All four drugs had both a relaxing and a lactic acid forming effect. The degree of relaxation tended to run parallel with the rise in lactic acid content.

Drugs inhibiting glycolysis totally abolished the relaxing effect of all sympathomimetic amines that were studied.

The concentrations of l-adrenaline, l-noradrenaline, dl-isopropyl-noradrenaline and l-ephedrine producing a maximal relaxing effect had a ratio of 1 : 2 : 10 : 350.

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From the Department of Pharmacology, Faculty of Medicine, Gothenburg University.

Effect of Calcium Ions Upon the Relaxing and Lactic Acid Forming Action of Adrenaline on Smooth Muscle.

By

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Received 7 March 1956.

The inhibiting effect of adrenaline upon smooth muscle seems to consist in the induction of lactic acid formation therein (MOHME-LUNDHOLM 1953). Substances which, by depressing or interfering with the carbohydrate metabolism, prevented formation of lactic acid in the smooth muscle, also inhibited the relaxing effect of adrenaline. Those that were found to possess this property were sodium fluoride, monoiodoacetic acid, glyceraldehyde, cupric chloride, sodium arsenate and sodium azid (MOHME-LUNDHOLM 1953). According to BOYER, LARDY and PHILLIPS (1943), calcium ions have an inhibiting effect on the enzyme phosphopyruvic kinase, which catalyzes the reaction phosphopyruvic acid + ADP $\rightleftharpoons$ pyruvic acid + ATP. This reaction is an essential stage in the formation of lactic acid. Theoretically it was probable, therefore, that calcium ions inhibited the lactic acid forming and the relaxing effect of adrenaline. The influence of calcium ions on the effects of adrenaline has been the subject of fairly numerous investigations (for references, *vide* TRENDELENBURG 1924). The results, however, have been extremely divergent; no clear picture can be obtained of the way in which different effects of adrenaline are influenced by an excess of calcium. TUROLT (1922) nevertheless found that calcium in excess converted the relaxing effect of adrenaline on guinea-pig uterus into a stimulating effect.

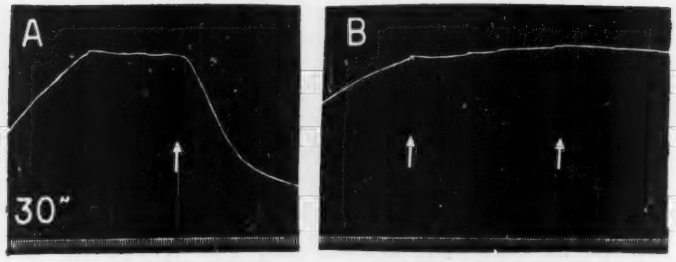

Fig. 1. Effect on bovine tracheal muscle of
A. Adrenaline 40 γ/20 ml.
B. $CaCl_2$ 200 mg/24 ml (first arrow).
Adrenaline 40 γ/24 ml (second arrow).

Method.

The experiments were performed on segments of bovine tracheal muscle (for details of the mode of preparation, *vide* MOHME-LUNDHOLM 1953). Three muscle preparations were used for each series of experiments: for determination, respectively, of the initial lactic acid content, and the effect thereon of $CaCl_2$ alone and $CaCl_2$ in combination with adrenaline. The preparations were suspended in a bath *ad modum* MAGNUS, at a temperature of 38° C. The Tyrode's solution was bubbled with 6.5 per cent CO_2 in O_2. The tone was recorded by means of an isotonic pen with a load of 40 g.

Results.

It was found that $CaCl_2$ in a concentration of $8.3 \cdot 10^{-3}$ (0.075 M) totally abolished the relaxing effect of adrenaline in a concentration of $2 \cdot 10^{-6}$ (figure 1). The effect on lactic acid formation in the tracheal muscle is shown in table I. It will be seen that neither calcium chloride nor a combination of calcium chloride and adrenaline had any effect on the lactic acid content. A corresponding dose of adrenaline alone increase that content by a mean figure of 26.1 ± 3.48 mg per cent in 11 experiments (MOHME-LUNDHOLM 1953). The sum of the separate effects of adrenaline and calcium chloride was found, on statistical analysis, to be significantly greater than the effect of the two substances in combination; *i. e.*, the calcium ions had depressed both the lactic acid forming and the relaxing action of adrenaline.

This inhibiting effect of calcium ions on each of the above-

Table I.

Effect of calcium chloride (200 mg/24 ml) and calcium chloride and adrenaline (40 γ/24 ml) on the lactic acid content of bovine tracheal muscle. The effect of adrenaline on the tone was totally abolished.

Basal value mg per cent	$CaCl_2$ tests Deviation from basal value mg per cent	$CaCl_2$-adrenaline tests Deviation from basal value mg per cent
42.4	5.2	4.8
38.5	— 4.0	— 11.5
31.2	3.0	0.1
35.8	— 3.2	— 0.3
33.5	— 1.0	— 4.2
43.5	1.9	1.6
35.5	1.6	— 0.6
29.8	1.3	3.4
30.0	7.9	— 2.9
31.2	— 1.8	— 5.2
Mean	1.1 ± 1.17	-1.5 ± 1.49
	$0.5 > P > 0.4$	$0.5 > P > 0.4$

$CaCl_2$ tests + adrenaline tests — $CaCl_2$-adrenaline tests $= 28.7 \pm 3.92$
$P < 0.001$.

mentioned actions of adrenaline lends further weight to the theory that the relaxing effect is a result of the lactic acid forming effect. The point at which calcium exerts its action in this respect is probably the enzyme phosphopyruvic kinase; at all events, no other calcium-susceptible enzyme in the glycolytic process is known.

Summary.

In experiments on bovine tracheal muscle, it was found that calcium ions in excess inhibited both the relaxing and the lactic acid forming effect of adrenaline.

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From the Institute of Biochemistry, University of Copenhagen.

A Vitamin B_{12}-binding Factor Formed in Cultures of Euglena gracilis var. bacillaris.

By

H. P. ØSTERGAARD KRISTENSEN.

Received 10 March 1956.

It has been previously reported (KRISTENSEN 1955) that the vitamin B_{12}-requiring alga *Euglena gracilis var. bacillaris* produces a thermolabile factor which inhibits the growth of the Euglena cells. This inhibition is found only when vitamin B_{12} is a limiting factor for growth and may therefore be due to the excretion of a substance which binds the vitamin in the medium and makes it unable to be utilized by the Euglena cells. The validity of this hypothesis has been examined in this investigation by testing the ability of supernatants from Euglena cultures to prevent the uptake of vitamin B_{12} by E. coli cells.

Methods.

Many strains of wild-type E. coli absorb very large amounts of vitamin B_{12} although they grow equally well without this vitamin (DAVIS and MINGIOLI 1950). This uptake of vitamin B_{12}, however, is inhibited by "intrinsic factor" preparations, presumably owing to the formation of a complex of vitamin B_{12} and a protein (HOFF-JØRGENSEN 1952 a, BURKHOLDER 1952).

HOFF-JØRGENSEN, SKOUBY and ANDRESEN (1952 b) as well as HOFF-JØRGENSEN and LANDBOE-CHRISTENSEN (1953) have described a

The present investigation was carried out with the support of the *Danish State Research Foundation*.

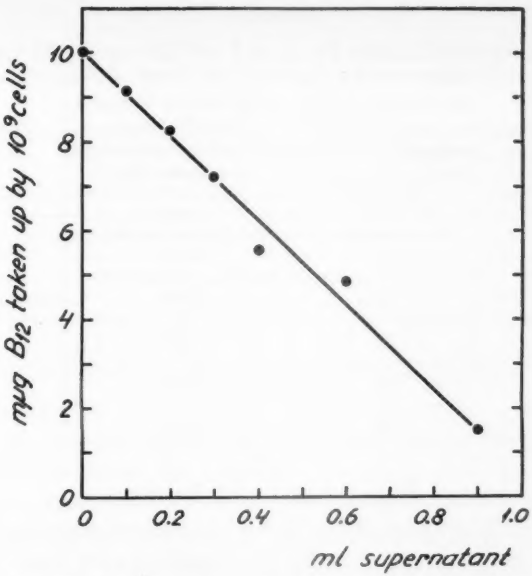

Fig. 1. The effect of different amounts of Euglena supernatants on the B_{12}-uptake by E. coli E 35. Supernatant from a 13 days old Euglena culture (per 2 ml medium: 0—0.9 ml of supernatant, 10 mμg B_{12}, 10^9 coli cells; $2^1/_2$ hrs.; 30° C.)

procedure for the assay of the B_{12}-binding capacity of "intrinsic factor" preparations, using a wild-type E. coli strain, coli E 35. The modification of this procedure described below has been used here in studying the B_{12}-binding effect of supernatants from Euglena cultures.

Euglena gracilis was cultivated as recommended by Ross (1952). Supernatants from the cultures were obtained by aseptical centrifugation.

E. coli E 35 was cultivated as described by HOFF-JØRGENSEN et al. (1952 b, 1953).

Procedure. In sterile centrifuge tubes were pipetted: 10^9 E. coli cells suspended in 1.0 ml coli medium; 0.1 ml of a solution of vitamin B_{12} containing 100 mμg B_{12}/ml; varying amounts of supernatant from Euglena cultures; and coli medium added to a total volume of 2 ml. The tubes were shaken for $2^1/_2$ hrs. at 30° C. After centrifugation the amount of vitamin B_{12} in the medium, *i. e.* the amount of vitamin B_{12} which had not been absorbed by the E. coli cells, was determined microbiologically with Lactobacillus leichmannii 313 as assay organism (HOFF-JØRGENSEN 1954).

Table I.

Effect of supernatants from Euglena cultures of different ages on the B_{12}-uptake by E. coli E 35.

(Per 2 ml medium: 0.5 ml supernatant, 10 mμg B_{12}, 10^9 coli cells; $2^1/_2$ hrs.; 30° C.)

Age of Euglena supernatant days	Unabsorbed B_{12} (remaining in the medium) mμg
5	1.105
9	3.370
12	4.845
16	5.990
43	8.670

Experiments and Results.

1. The effect of different amounts of Euglena supernatant on the amounts of vitamin B_{12} taken up by E. coli.

The procedure was as described above. 0.0—0.9 ml of supernatant from a 13 days old Euglena culture was used. A control experiment was made with a sample of the Euglena supernatant heated for 15 minutes at 100° C. to destroy the thermolabile factor.

The amounts of vitamin B_{12} taken up by the coli cells are inversely proportional to the amounts of Euglena supernatants added to the coli suspension, *i. e.* the amounts of "bound" vitamin B_{12} are directly proportional to the amounts of Euglena supernatant (Fig. 1). The control experiment showed that the heated sample of the supernatant had no inhibitory effect on the B_{12}-uptake by the coli cells.

2. The effect of supernatants from Euglena cultures of different ages on the B_{12}-uptake by E. coli.

The experiment was made with 0.5 ml of supernatants from 5—43 days old Euglena cultures.

The B_{12}-binding capacity of a supernatant increases gradually with the age of the Euglena culture from which the supernatant is obtained (Table I). One ml of supernatant from a five days old Euglena culture "binds" 2.21 mμg vitamin B_{12}; one ml of

Table II.

Binding of vitamin B_{12} by Euglena supernatant (supernatant from a 9 days old Euglena culture, dialysis for 24 hours, cellophane membrane, running water).

Within membrane	Vitamin B_{12} found within membrane after dialysis mμg
5 ml supernatant, 50 mμg vit. B_{12}, water to 20 ml	35.5
As above but with the supernatant *steamed* for 15 minutes	6.0

supernatant from a 43 days old Euglena culture "binds" 17.34 mμg B_{12}.

3. Examination of the dialyzability of the Euglena supernatant-vitamin B_{12} complex.

Supernatant from a nine days old Euglena culture was used. Fifteen ml tap water containing 50 mμg B_{12}, and 5 ml of the Euglena supernatant were placed in a cellophane tube and dialyzed against running tap water for 24 hrs. The amount of vitamin B_{12} remaining in the tube was then determined by the Lactobacillus method. A control experiment was carried out as above with 5 ml of a sample of the supernatant heated for 15 minutes at 100° C.

The Euglena supernatant contains a thermolabile factor which combines with vitamin B_{12} and thus renders it non-dialyzable through cellophane (Table II).

Discussion.

The experiments presented in this study show that supernatants from centrifuged cultures of the vitamin-B_{12}-requiring alga *Euglena gracilis var. bacillaris* contain a thermolabile factor which prevents the vitamin B_{12}-uptake by a wild-type E. coli, coli E 35. The amount of the factor present in the Euglena supernatants increases with the age of the Euglena cultures. It is probably an excretion product of Euglena cells and the amount formed will therefore depend on the number of the Euglena cells and the time passed since the inoculation of the medium.

The amounts of Euglena supernatants used in the above experiments do not inhibit the growth of E. coli E 35 in the coli medium.

There is a direct proportionality between the amount of Euglena supernatant added to the coli medium and the amount of vitamin B_{12} which is prevented from being taken up by the coli cells. These findings suggest that the factor in the Euglena supernatants acts on the vitamin and not on cells.

The dialysis experiment shows that the thermolabile factor described above in some way combines with the vitamin B_{12} and forms a complex which is not dialyzable through cellophane. The factor — as regards its B_{12}-binding effect, its thermolability, and its non-dialyzability — resembles the "intrinsic factor" of CASTLE, but at present it is not known whether this factor has any effect on the absorption of vitamin B_{12} in patients suffering from pernicious anæmia.

It is strange that a culture of the B_{12}-requiring *Euglena gracilis* can grow at all in a medium containing a B_{12}-binding substance. However, when Euglena cells are placed in a medium containing vitamin B_{12} in limiting amounts, the cells will very rapidly (in a day or two) absorb all the vitamin and store it intracellularly. — At this time the absorption can take place because no or very small amounts of the B_{12}-binding factor have been formed. — After the absorption of the vitamin, the Euglena cells can go on dividing in spite of the increasing amounts of the B_{12}-binding factor formed in the medium because they can now use their intracellular store of vitamin B_{12}.

FORD, GREGORY and HOLDSWORTH (1955) have recently found that extracts of the B_{12}-requiring protozoon *Ochromonas malhamensis* contain a substance which combines with the vitamin and renders it non-dialyzable through cellophane. The same or a similar substance was also found in the supernatants from Ochromonas cultures.

Summary.

Supernatants from centrifuged cultures of the vitamin B_{12}-requiring alga, *Euglena gracilis var. bacillaris*, contain a thermolabile factor which combines with the vitamin. The complex of vitamin B_{12} and the "Euglena factor" thus formed can not be taken up by the wild-type E. coli E 35 and is non-dialyzable through cellophane. The amount of the factor in the Euglena supernatants increases with the age of the Euglena cultures and is probably an excretion product of the Euglena cells. The factor, as regards its B_{12}-binding effect, its thermolability, and its non-

dialyzability, resembles preparations of the "intrinsic factor" of CASTLE.

Acknowledgement.

The author wishes to express his thanks to Professor RICHARD EGE, for excellent working conditions, to Dr. E. HOFF-JØRGENSEN who has assisted him with very valuable critical advice and to Dr. B. KING for help in preparing the English MS.

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From the Institute of Neurophysiology, University of Copenhagen.

Conduction Velocity and Amplitude of the Action Potential as Related to Circumference in the Isolated Fibre of Frog Muscle.

By

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Received 17 March 1956.

GASSER and ERLANGER's (1927) deduction of proportionality between diameter and conduction velocity in myelinated nerve fibres inaugurated more direct investigations of this correlation both in myelinated and unmyelinated nerve fibres. In small myelinated nerve fibres of the frog BLAIR and ERLANGER (1933) calculated the conduction velocity of the action potential to vary as the square of the diameter. HURSH (1939) and GASSER and GRUNDFEST (1939) interpreted their results to indicate a straight line relationship in the largest myelinated nerve fibres of the cat. In unmyelinated nerve fibres results are no less diverging. For the isolated giant axon of the squid PUMPHREY and YOUNG (1938) found a square root function; and HODES (1953) a linear relationship, as did GASSER (1950) in the unmyelinated C fibres of the cat.

The relationship in muscle has not been investigated systematically but would be expected to be analogous to that in unmyelinated nerve (KATZ 1948). The conflicting evidence as to the conduction velocity—fibre diameter relationship in nerve fibres and the paucity of evidence as to this relationship in muscle fibres suggested the present study.

Some of the previously mentioned conclusions as to the correlation between fibre diameter and conduction velocity were based

[1] Working under a grant from the Rask-Ørsted Foundation.

on the assumption of a correlation between amplitude of the action potential and fibre diameter. Thus GASSER and ERLANGER (1927) in reconstructing the combined nerve action potential assumed that the spike amplitude varied with the square of the fibre diameter. BLAIR and ERLANGER (1933) in frog fibres and ZOTTERMAN (1937) in mammalian fibres calculated also a square relation between amplitude and fibre diameter. GASSER and GRUNDFEST (1939) on the other hand obtained better fit with the assumption of a linear relationship between amplitude and diameter. The diverging opinions as to the correlation between action potential amplitude and fibre diameter made it desirable to investigate this relationship directly as well.

Two considerations made it essential to use isolated fibres. The elliptical cross section displayed by most fibres necessitated their isolation for circumference measurements. Moreover in view of the fact that conduction velocity in nerve has been shown to depend on the external resistance (HODGKIN 1939), it was considered important to study the fibre in a uniform medium of low resistance, *i. e.* in a large volume of Ringer's solution. Such conditions do not obtain with the fibre in situ.

Methods.

The experiments were performed on isolated fibres from m. semitendinosus in *Rana temporaria* (summer and winter frogs). The following quantities were measured in this study:

1. *Fibre size.* Since most fibres have an elliptical cross section, it was impracticable to characterize their size in terms of diameter. Therefore, the circumference was used as the standard dimension.

2. *Conduction velocity of the action potential.* In principle conduction velocity was determined by stimulating the fibre at its one end and recording the action potential at two different points along the fibre. From the distance between the electrodes and the time interval between the action potentials, the conduction velocity was calculated. The time interval was defined as the time between the action potentials measured at the intersection of the steepest potential deflection with the base line.

3. *Amplitude of the action potential.* The amplitude was measured from peak to peak. It was recorded externally in a large volume of Ringer's solution. Since amplitude varies considerably with the distance between fibre and recording electrode it was necessary for the comparison of the amplitudes of different fibres that this distance be well defined.

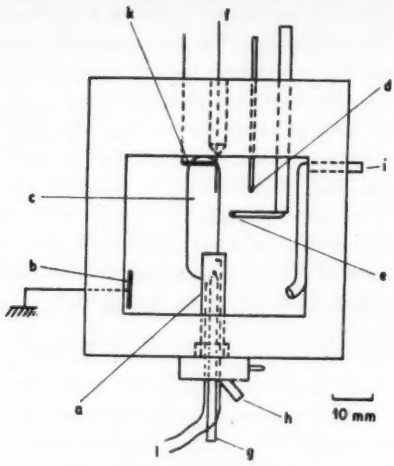

Fig. 1. Experimental chamber.

a. Stimulation chamber (details see fig. 2).
b. Earth electrode.
c. Glass window in the bottom of the chamber.
d. Thermistor for automatic temperature control.
e. Thermistor, adjustable in position, recording the temperature.
f. Glass rod to which the fibre was attached.
g. Movable perspex rod.
h. Perspex tube through which the stimulation chamber was filled with Ringer's solution.
i. Tube for bubbling a mixture of 1 per cent CO_2 and 99 per cent O_2 through the Ringer's solution.
k. Reference electrode.
l. Stimulating electrodes.

Technique of fibre isolation. The semitendinosus muscle was dissected from a previously curarized frog (50 μg curarine per gram frog), and placed in a preparation dish in Ringer's solution, to which curarine (3×10^{-6} g tubocurarine per ml) had been added to prevent contraction of the muscle fibres from pulling on the nerve twigs, since these contractions seemed to leave the fibres unsuitable for experimental use. Suitable fibres were selected during preparation by testing contractility with the dissecting scissors connected to the cathode of the stimulator. The fibres were then isolated under a binocular microscope (10—40 $\times$ magnification) provided with a watercooled stage. It was important to avoid injury to the sarcolemma inflicted by pulling at the abundant connective tissue strands interconnecting adjacent fibres (Buchthal and Knappeis 1940) (fig. 3).

After isolation, the fibre was transferred to the experimental chamber and maintained at a length at which the fibre was just taut.

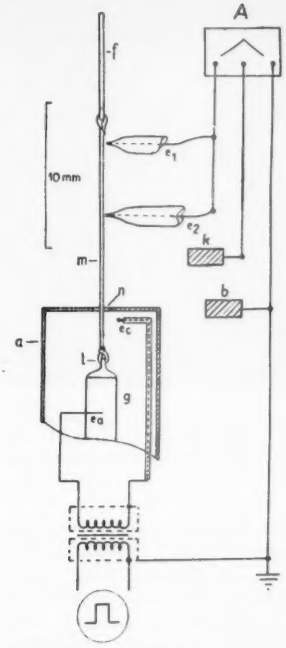

Fig. 2. Stimulus isolation chamber and arrangement for recording.

A. Differential amplifier.
a. Stimulus isolation chamber.
b. Earth electrode.
e_A. Stimulating electrode, anode.
e_C. » » , cathode.
e_1, e_2. Recording electrodes.
f. Glass rod.
g. Movable perspex rod.
k. Reference electrode.
l. Platinum loop at the end of the perspex rod.
m. Muscle fibre.
n. Opening of 0.7 mm diameter for the muscle fibre.

The experimental chamber (fig. 1) consisted of a double walled perspex box measuring 50 × 50 × 20 mm. The isolated fibre was placed in the centre of the chamber which was filled with Ringer's solution. The fibre was attached at one end by its tendon to the glass rod (fig. 1, f) and the other end by its tendon to the platinum loop at the tip of the movable perspex rod inside the stimulation chamber (fig. 1, g and fig. 2).

Recording electrodes. The recording electrodes consisted of glass capillary tubes containing Ringer's solution and a platinum wire inserted to within 50 μ from the tip. The platinum wire was connected

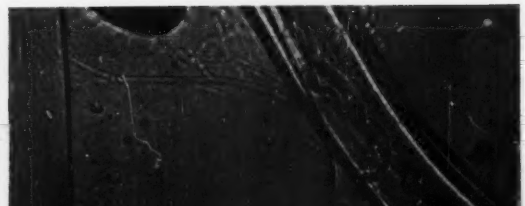

Fig. 3. Connective tissue strands between two adjacent fibres. Note the protrusion of the fibre to the right, caused by tension on the strand. Further tension leads to laceration of the sarcolemma.

to the cathode follower of the differential amplifier. Such an electrode had a noise level of about three db over the noise level of the amplifier. For recording action potential amplitudes, one electrode was placed at a given distance from the fibre and replaced after each contraction. The amplitude was determined as a mean value of four successive recordings with an average variation of 2.5 per cent. For recording conduction velocity, two electrodes were used, mounted on the same arm of a micromanipulator with a distance of three to five mm between the tips. This distance was measured by means of a measuring microscope with an accuracy ± 0.05 mm. The two action potentials were recorded on the same beam of the oscilloscope; both recording electrodes were connected to the input of the same amplifier, resulting in a voltage division which caused a decrease both of the action potential amplitude and of the noise level.

Electrode diameter. The amplitude of the recorded action potential was affected by the diameter of the recording electrode. The amplitude recorded from a fibre with a circumference of about 300 μ was 8—10 per cent lower with a 100 μ than with a 40 μ electrode. For a fibre with a 200 μ circumference the difference was 10—12 per cent. No appreciable variation in action potential amplitude with electrode size occurred in fibres with a circumference of more than 300 μ as long as the electrode diameter was less than 100 μ. With an electrode diameter less than 100 μ the relationship between action potential amplitude and electrode—fibre distance was the same for fibres of all circumferences. In the present study electrodes with an internal diameter of 30—50 μ were used. The error in amplitude measurements due to variations in electrode size in this range did not exceed 10 per cent for the smallest

fibres. Electrodes small enough to exclude this source of error entirely could not be used because of their high noise level.

Impedance. The impedance of the electrodes used for extracellular recording did not exceed 50,000 Ohms. With the input impedance of the amplifier of 100 Megohms in parallel with 1 $\mu\mu$F, the electrode impedance was so low as to cause no appreciable distortion of the signal at an upper limiting frequency of 10^4 c.p.s.

Stimulating electrodes and arrangement for preventing stimulus escape. The stimulating electrodes consisted of silver wires; the cathode was insulated with exception of a 1 mm^2 stimulating area of the tip. The distance between the two stimulating electrodes was 5 mm and the stimulus was applied to the one end of the fibre. Single square wave impulses of adjustable strength and duration were used for stimulation. In most experiments the duration was 220 μsec. and the strength just above threshold. The stimulus triggered the sweep of the cathode ray oscilloscope with a suitable delay.

To prevent diffuse spread of the stimulating current through the Ringer's solution to the recording electrodes, the stimulating electrodes were carried in a perspex tube (8 × 15 mm). The Ringer's solution inside the tube made contact with that of the main chamber only via the opening (n, fig. 2) for the fibre. For a further reduction of the stimulus artifact the stimulating electrodes were connected to the secondary coil of a doubly shielded transformer with a low capacity to ground (Buchthal, Guld and Rosenfalck, 1955 a).

Measurement of circumference. The circumference of an ellipse with axes a and b can be approximated by the expression:

$$\frac{\pi}{2}(a + b)$$

When $a \leqq 2\,b$, $\frac{\pi}{2}(a + b)$ will differ maximally by three per cent from the true circumference. The slack fibre was placed in the preparation chamber (fig. 1), where it could be rotated, by means of the perspex and glass rods to which the tendons were attached (f and g, fig. 1). The two transverse axes of the fibre were measured by means of an eye piece micrometer with movable cobweb at a magnification of 150 times. The error of this determination did not exceed three per cent. Nor was the variation from point to point in the portion of the fibre investigated more than three per cent. In most instances the circumference was measured at a point midway between the two recording electrodes.

Amplifier. The amplifier used was push pull and with RC coupling. For intracellular measurements it could be converted to DC. For 1 c.p.s. and 30,000 c.p.s. the frequency curve showed a decrease of 3 db. The input impedance was 100 Megohms in parallel with 1 $\mu\mu$F. The differential amplification (in phase signal rejection) of the amplifier itself was more

than 20,000 over the entire frequency range. With the input connected to the recording electrodes in Ringer's solution the differential amplification was more than two hundred up to 10^4 c.p.s.

To measure the noise level a sinusoidal input voltage was applied of such a strength, that the output voltage exceeded the noise level of the amplifier by three db. By this method it was found to be 2 μV r.m.s. up to 10^4 c.p.s.

Recording. The action potentials were recorded from a DuMont double beam oscilloscope (type 279), by means of a Fairchild camera on 35 mm film. The negatives were enlarged about seven times for measurements.

The Ringer's solution consisted of:

NaCl	6.7 g/l
KCl	0.2 »
$CaCl_2$(anhydr.)	0.4 »
glucose	0.2 »
dextrane[1]	30.0 »

1.6 ml 2.5 per cent $NaHCO_3$ was added to the solution and a gas mixture consisting of 1 per cent CO_2 and 99 per cent O_2 bubbled continuously through it giving a pH of 7.2—7.4 (cf. STEN-KNUDSEN 1953).

Temperature control. The Ringer bath was kept at constant temperature by circulating fluid of a given temperature between the two perspex layers, forming the bottom of the chamber. The rate of flow was controlled by a thermistor inside the chamber which triggered a circulating pump via an electronic relay. The gas mixture bubbling through the Ringer's fluid in the chamber insured equal temperature distribution. Another thermistor close to the muscle fibre recorded the temperature of the Ringer's solution on a galvanometer. The temperature was maintained constant within 0.2°C.

Results.

A. Conduction Velocity of the Action Potential.

1. *Conduction velocity as a function of time after isolation.*

There was a continuous decrement in both the conduction velocity and the amplitude of the action potential with time after preparation. Although the change was small it was necessary in order to compare results obtained from different preparations to extrapolate the values of the conduction velocity measured at a given time to zero time after isolation. After two hours the velocity averaged 95—90 per cent of the value at one hour; 85—75

[1] The intrinsic viscosity as given by the manufacturer (Pharmacia, Uppsala) was 0.307; 30 g dextrane per litre give a colloid osmotic pressure of 110 mm H_2O.

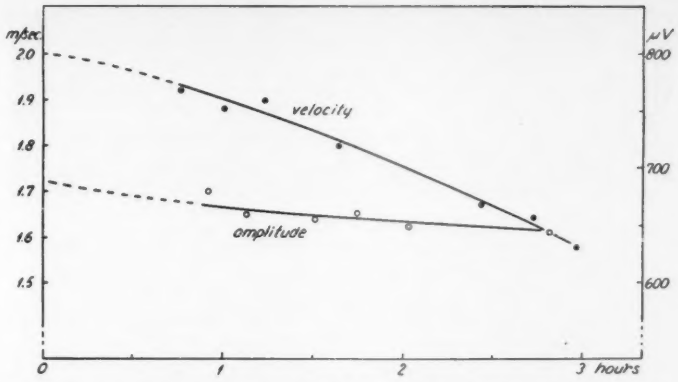

Fig. 4. Decrement with time after isolation in conduction velocity and amplitude of the action potential recorded from an isolated muscle fibre in a large volume of Ringer's solution, 20°C. Extrapolation of the curve to zero time is indicated by the broken lines.

per cent at three hours. In fig. 4 is given the decrease in conduction velocity and amplitude of the action potential for a fibre stimulated only 30 times in the course of three hours. With more frequent stimulation there was a more rapid decrease in conduction velocity, and the number of tests was therefore kept to a minimum. Usually at 4—6 hours after isolation with repeated contractions the fibres ceased to give a propagated potential and a full contraction. In the microscope it could be seen that the contractions which occurred were localized to the cathode. That this may have been due to a fall in the membrane potential below that necessary for propagation (JENERICK and GERARD 1953) is indicated by three experiments in which the intracellularly measured potential at this time was found to be only 30—50 mV.

To determine whether the change in conduction velocity with time was due only to preparation damage, some experiments were carried out on *whole muscle*. The semitendinosus muscle (500 fibres) was kept in Ringer's solution at equilibrium length for 19 hours at constant temperature and pH. Within this time the propagation velocity decreased to half its initial value. During the first 3—4 hours there was a change in the form of the potential wave, but not in the conduction velocity of the action potential as a whole. In a muscle with fewer fibres (m. extensor longus dig. IV, 50 fibres) conduction velocity changed more rapidly.

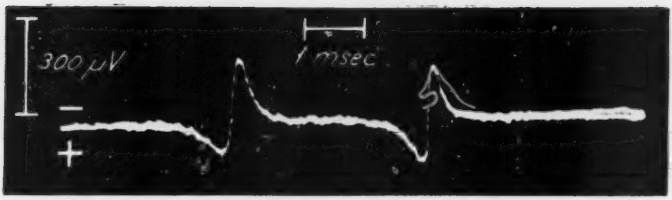

Fig. 5. Action potentials from an isolated muscle fibre in Ringer's solution, 20°C. The fibre had a circumference of 294 μ and the electrodes were at a distance of 100 μ from the fibre.

The first 3—4 hours it had a decrement similar to that of an isolated fibre, but thereafter the decrement was less.

The cause of the changes with time is presumably a change in ion balance, which occurs with the fibre in an artificial environment. At least for nerve fibres, a continuous potassium leakage and a gain in sodium have been found with time after isolation (KEYNES and LEWIS 1951). The potassium leakage is associated with a change in membrane properties (HODGKIN and HUXLEY 1947). As reported for sepia axon (WEIDMANN 1951) no external medium was found which would prevent the alteration in the fibre.

2. *Conduction velocity as related to muscle fibre circumference.*

The action potentials of the single fibre, recorded by two electrodes four mm apart and connected to a common input and with a common reference, are shown in fig. 5. The first deflection was positive and the duration of the diphasic response was about 1.5 to 2 msec. (20°C). The conduction velocity of isolated fibres in Ringer's solution is shown as a function of fibre circumference in fig. 6 I and II. The values to the right are corrected for the decrement in conduction velocity with time after isolation (p. 20—21) and the magnitude of this correction is presented in tables 1 and 2. The two sets of points (I and II) in fig. 6 give two series of experiments, one comprising 15 fibres, performed in the period March and April, and the other comprising 19 fibres in September through December. The spring experiments show much less variation and a slightly higher conduction velocity. In both series, the correlation between conduction velocity and circumference was very close to rectilinear, an ex rapolation of the curve passing through zero. The points could not be fitted to a square root function.

Table 1.

Conduction velocity in muscle fibres from spring frogs at 20°C. Uncorrected values and values corrected for the decrement with time after isolation.

Experiment No.	Time after isolation minutes	Circumference μ	Velocity uncorrected m/sec.	Velocity corrected m/sec.
1.....	15	385	2.97	3.10
2.....	30	335	2.60	2.73
3.....	30	331	2.42	2.53
4.....	6	304	2.20	2.28
5.....	26	296	2.22	2.30
6.....	20	294	2.24	2.31
7.....	25	289	1.60	1.80
8.....	27	264	2.20	2.28
9.....	35	259	1.75	1.80
10.....	30	244	1.70	1.79
11.....	10	226	1.70	1.74
12.....	30	225	1.80	1.85
13.....	48	202	1.20	1.40
14.....	45	201	1.22	1.60
15.....	15	161	1.14	1.25

Table 2.

Conduction velocity in muscle fibres from autumn frogs at 20°C. Uncorrected values and values corrected for the decrement with time after isolation.

Experiment No.	Time after isolation minutes	Circumference μ	Velocity uncorrected m/sec.	Velocity corrected m/sec.
1.....	35	428	2.5	2.75
2.....	27	386	2.25	2.30
3.....	22	372	2.30	2.40
4.....	37	360	2.07	2.20
5.....	30	360	2.15	2.28
6.....	44	354	1.92	2.05
7.....	25	333	2.22	2.40
8.....	24	319	2.05	2.15
9.....	30	315	1.65	1.73
10.....	30	315	1.93	2.25
11.....	38	290	1.85	2.0
12.....	38	285	1.90	2.04
13.....	25	285	1.60	1.70
14.....	38	280	2.0	2.10
15.....	30	257	1.74	1.81
16.....	37	250	2.0	2.10
17.....	30	210	1.43	1.50
18.....	30	159	0.95	1.0
19.....	15	148	1.10	1.12

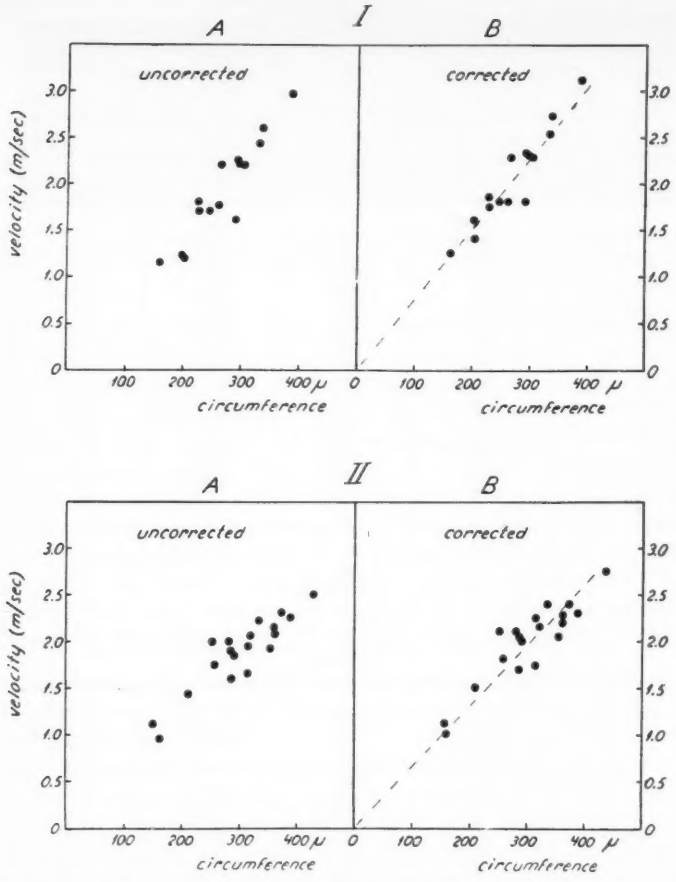

Fig. 6. Conduction velocity of the action potential plotted against the circumference of the muscle fibre. I. Spring frogs. II. Winter frogs 20°C.; A, uncorrected values; B, values corrected for the decrement with time after isolation.

The maximum values of circumference represented the largest muscle fibres found in *Rana temporaria* in this study and correspond to a circular fibre with a diameter of 135 μ. The minimum circumference for which conduction velocity could be measured corresponds to a circular fibre with 45 μ diameter and did not represent the smallest fibres present. Fibres ranging between

10 and 20 μ in diameter were often seen, but their isolation without damage was not possible.

B. The Amplitude of the Action Potential.

When the action potential was recorded outside the muscle fibre, its amplitude was influenced by electrode size, the distance between electrode and fibre, and whether it was placed near the flat or the rounded surface of the fibre. These factors are dealt with below and a further analysis of the externally and intracellularly measured action potential will be published.

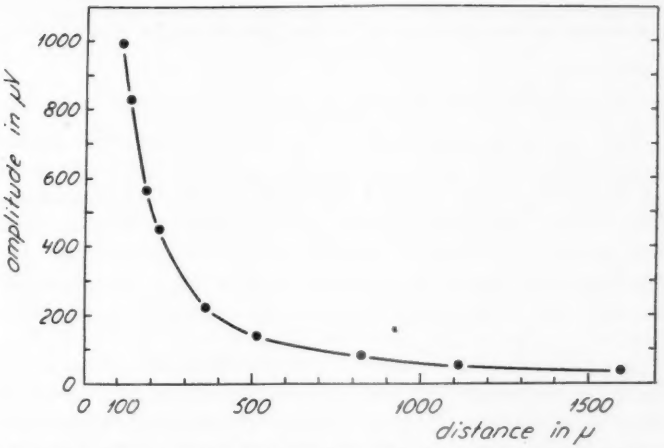

Fig. 7. Peak to peak amplitude of the action potential as a function of the distance between electrode (40 μ internal tip diameter) and the midpoint of a circular fibre (circumference 350 μ) 22°C.

1. *Amplitude as a function of the distance between muscle fibre and recording electrode.*

The peak to peak amplitude of the action potential decreased with increasing distance between fibre and recording electrode (fig. 7). Within a distance of 200 μ from the fibre, the amplitude fell steeply with increasing distance. It reached one tenth of the maximally measured value at a distance of about 600 μ. At larger distances there was little change in amplitude.

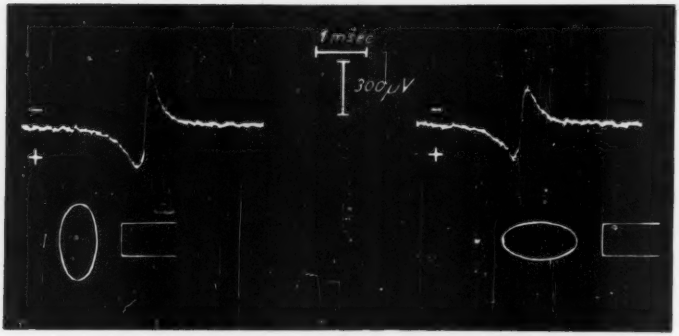

Fig. 8. Action potentials recorded by a 45 μ electrode at a distance of 22 μ from the surface of a fibre with diameters of 45 and 90 μ. Left: action potential recorded from the flat surface of the fibre, right: action potential from the rounded surface of the fibre. 19°C.

2. *The influence of the shape of the fibre on the recorded amplitude.*

With the electrodes used (30—50 μ diameter), the action potential amplitude was greater when the recording electrode faced the flat surface than when it faced the rounded surface of the fibre at identical distances. For a fibre with an elliptical cross section where the largest diameter was twice the smallest, the amplitude was about 20 per cent smaller when the recording electrode faced the rounded surface at a distance of 20—40 μ from the surface of the fibre than when it faced the flat surface. This phenomenon is in fact an expression of the variation in recorded amplitude with the size of the electrode relative to fibre circumference (cf. p. 18). Since the recording electrodes as far as possible were placed facing the flat side of the fibre, the recorded amplitude for the smallest fibres (150 μ circumference) is estimated to be 10 per cent smaller than the amplitude which would be obtained with an electrode of very small diameter. With increasing circumference the effect decreases and is zero for fibres with a circumference greater than 300 μ.

3. *The amplitude as a function of time after isolation.*

As in the case of conduction velocity, the amplitude of the action potential decreased progressively with time after isolation of the fibre (fig. 4). The decrement in amplitude amounted to 3—5 per cent per hour — less than the decrement in conduction velocity. To compare the amplitude measurements from different fibres

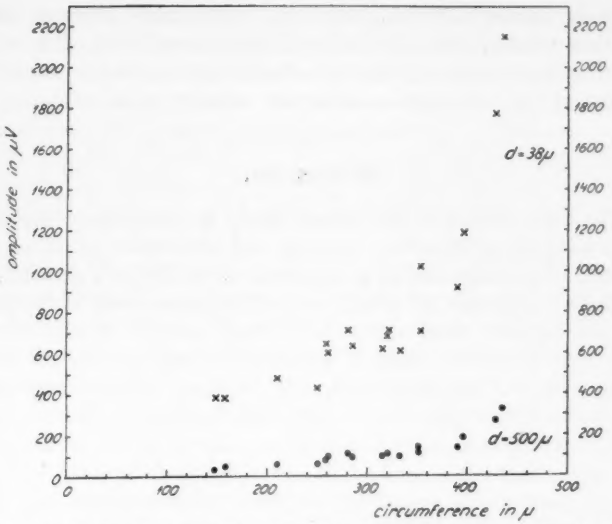

Fig. 9. Amplitude of the external action potential as a function of muscle fibre circumference; d = distance between fibre and recording electrode. 18 fibres. 20°C. Upper curve: amplitude at 38 μ from the surface of the fibres. Lower curve: amplitude at 500 μ from the midpoint of the fibre.

the value at zero time after isolation was obtained for each fibre by extrapolation along the decremental curve.

4. *Amplitude as a function of fibre circumference.*

In view of the dependence of action potential amplitude on the distance and the orientation of the recording electrode with respect to the fibre, standard conditions had to be observed to allow an estimation of the relation between action potential amplitude and fibre circumference in different experiments. In fig. 9 is given action potential amplitude as a function of fibre circumference with the recording electrode at a constant distance from the fibre surface (38 μ). Furthermore the amplitude—circumference relationship was calculated as it would appear at a distance of 500 μ from the midpoint of the fibres, assuming the fibres to have a cylindrical form.

The amplitude increased with increasing fibre circumference, being relatively greater for fibres of large circumference (fig. 9). The values are corrected for the decrement with time after isolation. In no case did the correction exceed five per cent of the

actually measured value. Since the relationship between conduction velocity and fibre circumference was linear, this relationship between amplitude and circumference was also an indication of the amplitude—conduction velocity relationship.

Discussion.

The main finding of the present study is a rectilinear relationship between propagation velocity and circumference of single muscle fibres immersed in a large volume of Ringer's solution.

Modern concepts of conduction of impulses over nerve and muscle suppose re-excitation from local current circuits (HERMANN, 1872) as the cause of conduction along a structure with properties of a core conductor. The original two element core conductor model (MATEUCCI, 1863) was modified by GRÜNHAGEN (1873) to a three element model. His conception of a sheat between the inner and outer conductors and differing from them in electrical properties is essentially the same cable structure assumed in modern models. The first attempt to explain propagation velocity was that of LILLIE (1914) who suggested that the velocity was inversely proportional with the time occupied by the rise of the action current to the critical stimulating point and proportional with the maximal distance at which the potential causes stimulation. CREMER (1923) included the internal and external conductance of a core conductor model in his expression for propagation velocity. On the basis of BLAIR's (1932) mathematical expression for the excitation process, RASHEVSKY (1933) calculated the properties of a model consisting of a network of resistors. This formula is equivalent to that of RUSHTON (1937) who described propagation velocity (V) in terms of a resistance-capacitance model according to the following expression:

$$V = \frac{\lambda \cdot L}{a}$$

where L is the characteristic length, a the time constant of the membrane and λ the safety factor. The safety factor indicates that the action potential is of a size exceeding the value necessary for stimulation. The smaller the margin, the less the propagation velocity.

While excitation in RASHEVSKY's and RUSHTON's models was conceived as the sudden building up of an electromotive force,

OFFNER, WEINBERG and YOUNG (1940) worked out a theory of propagation velocity considering excitation to be associated with a sudden depolarisation (BERNSTEIN, 1912). In a large volume of Ringer's solution where the external resistance is small compared with the internal, propagation velocity is given as:

$$V = \frac{\lambda}{\sqrt{2R_m}} \cdot \frac{\sqrt{r}}{C_m \sqrt{R_i}}$$

where λ is the safety factor, R_m the resistance of the active membrane in Ωcm^2, C_m the membrane capacity per unit area in $\mu F/cm^2$, R_i the specific internal resistance and r the fibre radius. With the assumptions mentioned below this expression indicates that the propagation velocity varies with the square root of the fibre diameter (OFFNER, WEINBERG and YOUNG 1940; RUSHTON 1951, HODGKIN 1954).

The assumptions are:

1. The safety factor is constant.
2. The electrical parameters of the membrane per unit dimension are independent of fibre diameter.
3. The internal action current is spread homogeneously over the cross section of the fibre.

The finding in the present experiments of a rectilinear relationship between propagation velocity and fibre diameter is in disagreement with the theoretical concept as outlined above; but it is not possible on the basis of present experimental evidence to determine with certainty the reason for this disagreement. A priori one might expect that one or more of the above conditions is not fulfilled. There is evidence that the electric properties of the membrane do in fact vary with diameter. Thus KATZ (1948) found that the specific membrane conductance and capacitance were greater for muscle fibres of large circumference than for small fibres. In order to correlate KATZ' findings on the membrane properties with our findings on propagation velocity, the following equation may be used:

$$V = K\left(\frac{1}{C_m} \cdot \frac{1}{R_m}\right)$$

where K is a proportionality constant and $C_m \cdot R_m$ the time constant of the membrane. Inserting KATZ' membrane constants for a fibre of 240 μ circumference ($R_m = 1{,}500\ \Omega cm^2$, $C_m = 6\ \mu F/cm^2$)

and our value for the conduction velocity for a fibre of the same size (1.8 m/sec.) (cf. fig. 6 I B), the proportionality constant can be determined to $K = 1.6 \times 10^4$. With this value of K and with KATZ' membrane constants for a muscle fibre with a circumference of 140 μ ($R_m = 4{,}000\ \Omega cm^2$, $C_m = 4.5\ \mu F/cm^2$), the conduction velocity calculated from the expression above is 0.9 m/sec. This value is very close to our finding of 1 m/sec. for a fibre of this size. The agreement lends support to the argument that the divergence of the present experimental findings from the calculated square root relationship is due to the fact, that the membrane properties vary with fibre circumference.

The present findings are in disagreement with those of ECCLES, KATZ and KUFFLER (1941) and of KATZ (1948) who studied propagation velocity in whole frog muscles. The range of propagation velocity in the earlier studies was narrower, 1—2.2 m/sec. (20°C) according to KATZ, 1.2—2 m/sec. (20°C) according to ECCLES, KATZ and KUFFLER. In addition, the values for maximum propagation velocity are different, the range being 1—3 m/sec. (20°C) in the present study. This disagreement may be due to the different experimental conditions, since the isolated fibres were immersed in a large volume of Ringer's solution whereby the external resistance was small as compared with the internal. It has been shown (BUCHTHAL, GULD and ROSENFALCK, 1955a, 1955b) that propagation velocity for fibres in situ is nearly constant in spite of a threefold variation in fibre diameter. The authors have accounted for their finding by considering that fibres in situ are surrounded by inactive fibres with high resistance which act as insulators. When the external resistance is of the same order of magnitude as the internal, the cable theory implies a considerable reduction in the variation of propagation velocity with diameter as well as an overall reduction in velocity.

The *amplitude* of the action potential varied according to the side of the fibre from which the potentials were led off. The amplitude was greater with the electrode facing the flat side of the fibre. Assuming that the muscle fibre membrane is homogeneous over its entire surface, and that current lines intersect the fibre membrane perpendicularly, the current lines would be denser at the flat than at the rounded side of the fibre. Since potential amplitude is proportional to the output of current through the fibre membrane, the variation in amplitude according to the surface facing the electrode is explicable on this basis.

In addition even if current density were identical for both the flat and the rounded surface of the fibre, the electrode would record a slightly smaller amplitude at the rounded surface, because a smaller part of the field over which the potential is averaged is close to the fibre.

The action potential amplitude was relatively larger for fibres of large than for fibres of small circumference. This finding is in disagreement with the rectilinear relationship between action potential amplitude and conduction velocity in unmyelinated C fibres of mammalian nerves where conduction velocity is proportional to circumference (GASSER 1950). Also in myelinated nerve fibres, most investigators agree that the relationship between potential amplitude and conduction velocity is linear (BLAIR and ERLANGER 1933, GASSER and GRUNDFEST 1939). Only ZOTTERMAN (1937) shows a figure where the amplitudes are relatively greater for the fast fibres.

The prerequisite for an interpretation of the amplitude—circumference relationship in terms of volume conductor theory (LORENTE DE NÓ 1947) is that the externally recorded amplitude is a measure of the amplitude of the intracellularly recorded potential. This has in fact been found and the findings will be described in detail in a separate publication.

Action potential amplitude was found to be relatively higher for fibres of large circumference than for small fibres. This indicates that large fibres have a relatively greater current output per unit area of the surface than small fibres. If the output of the action current per unit area of the fibre surface were constant for fibres of different circumferences, amplitude measured at a large distance from the fibre would be expected to vary rectilinearly with fibre circumference. Actually it increased more than linearly with increasing circumference (fig. 9, lower curve). Evidence has already been presented that the specific electrical properties of the membrane differ according to the fibre circumference and this was proposed to account for the linear variation in conduction velocity with fibre circumference. Adaptation of the velocity formula to conditions in muscle must await determination of the electrical constants of the membrane, the magnitude of the resting potential and the threshold of the action potential in relation to fibre circumference.

Summary.

1. Conduction velocity and amplitude of the action potential and fibre circumference were measured in isolated frog muscle fibres in a large volume of Ringer's solution.

2. Conduction velocity was found to be a linear function of fibre circumference.

3. The velocity in a fibre of 100 μ diameter (circumference 314 μ) at 20°C was 2.44 ± 0.05 m/sec. for spring frogs and 2.05 ± 0.07 m/sec. for winter frogs. The total range in conduction velocity at 20°C was 1.2—3.1 m/sec. for spring frogs and 1.0—2.75 m/sec. for winter frogs.

4. The amplitude of the action potential, both at constant distance from the fibre surface and at 500 μ from the midpoint of the fibre, was relatively greater in fibres with large than in fibres with small circumference.

5. The amplitude decreased steeply with electrode distance from the fibre up to 200 μ and reached one tenth of the maximally measured value at a distance of about 600 μ.

At equal distance the amplitude was 20 per cent smaller when the electrode faced the rounded side of an elliptical fibre, than when it faced the flat surface (large diameter twice the small).

6. The findings with respect to conduction velocity and amplitude as a function of circumference are interpreted to indicate that the specific electrical properties of the membrane differ according to fibre size.

Acknowledgments.

The author wishes to express his appreciation to Professor F. BUCHTHAL for suggesting the present problem and for his constant encouragement and advice and to Mr. CHR. GULD, E. E. whose technical and theoretical knowledge have been of invaluable assistance.

The work has been supported by the Michaelsen Foundation and the Rockefeller Foundation.

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The Critical Temperature in Naked Man.

By

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Received 12 March 1956.

A warm-blooded animal usually first compensates for a falling environmental temperature by gradually increasing the insulation while the metabolism remains at resting level. At the critical temperature insulation reaches its maximum, and from then on heat balance is obtained by increasing heat production. This increase has been found to be rather closely proportional to the body-to-air temperature gradient, such as would be expected from Newton's law of cooling.

The critical temperature is, accordingly, the lowest air temperature at which the animal can maintain its body temperature when the metabolic rate is at resting level. In tropical animals the critical temperature is 22° to 27° C. In arctic animals it varies from + 15° in the smaller forms to — 40° C or less in the larger animals. Clearly, therefore, the critical temperature is a fundamental index of the overall thermal adaptation of the animal (cp. SCHOLANDER et al., 1950; SCHOLANDER, 1955; KROG et al., 1955).

In naked man the critical temperature is known only for white man of normal indoor acclimation and is between 25° and 27° C. White man responds therefore as a tropical animal (DUBOIS, 1936; HARDY and DUBOIS, 1940). At lower temperatures shivering begins, and the metabolism increases. The quantitative relation between this increase in heat production and the body-to-air temperature gradient has not been investigated in man.

We shall present data on the critical temperature in five naked men and their metabolic response when the temperature is lowered below this point.

Method and Procedure.

A cold room was thermoregulated to within one degree. Air circulation was slight, only enough to keep the temperature even. The room was set at the proper temperature 12 hours before the run, so that the walls and all objects were at the same temperature. Humidity was between 45 % and 65 %. The subject sat on a 3/4-inch mesh fishnet sling stretched in the frame of a steel chair and pedaled from this position an ergometer wheel. The metabolic rate was determined by the CO_2 production[1] recorded by a respirometer placed outside the cold room (ERIKSON et al. 1951). Rectal temperature and room temperature were continuously recorded, as well as the work performed on the ergometer.

The subject, fasting for some hours beforehand, undressed except for shorts and shoes; after which he entered the cold room, sat down in the ergometer chair, and inserted a rectal thermocouple. At temperatures higher than 25° C he sat relaxed and comfortable in the net, resting his feet on the ergometer pedals. At lower temperatures he commenced to bicycle, adjusting his work subjectively to a level, such that he would barely avoid the appearance of goose pimples or shivering. In the beginning there was considerable hunting before the proper work level was found. Time was saved when the person already knew at the start approximately how fast to pedal. After half an hour of adjustment the subject inserted the mouthpiece connecting him with the respirometer, and the carbon dioxide output was recorded in three successive 2-minute periods, after which the mouthpiece was taken out. This was repeated every 15 minutes for one to two hours. The subject pedaled as evenly as possible, while in the cold room, whether breathing through the mouthpiece or not. Each person made two or three runs at each temperature.

The individual resting rate of CO_2 production was obtained at 27° to 30° C. Higher rates were expressed as percentages of this value.

Results and Discussion.

Five men[2] were tested, and the results were so similar that they will be considered together. At 26° and 27° the rate of CO_2 production was the same as at 30° C. At 17° the rate was for all individuals approximately doubled, at 6°, tripled, and at — 6° C, five times the resting rate.

[1] The gas exchange figures were obtained under or near steady state conditions. Tests showed that the CO_2 output gave the same relative results as the more cumbersome oxygen determinations.

[2] Age, weight (kg), and height (cm), respectively: 50, 74, 176; 35, 74, 170; 37, 73, 173; 40, 70, 179; 31, 78, 190.

In white man, as in tropical mammals, the critical temperature is, accordingly, approximately 25° to 27°, and from there down to 6° C the energy production increases approximately in proportion to the air-to-body gradient, as would be expected from Newton's law of cooling. At — 6° C the subjects became quite uncomfortable; two overshot the line of proportionality rather markedly. The rectal temperature remained in all cases constant within ± 0.4° C.

It is difficult to determine the critical temperature directly because of the large heat capacity of the human body and the consequent long time it takes to obtain equilibrium. The time factor is more favorable when the gradient is larger, such as at 5° C. It is easier, therefore, to calculate the critical temperature indirectly. This can be done by determination of the metabolic rate at rest and at 5° C. The point of intersection of a line representing resting level with a line connecting the rate at 5° C with zero rate at body temperature (37° C) will represent the critical temperature (Fig. 1).

To illustrate the effect of increased insulation on the critical temperature, we made determinations on one man dressed in winter sports clothing[1], bicycling at — 2° C. This gave a critical temperature of 14° by the intersection method, which was checked by determinations at 14° C.

In our experiments nearly all the heat generated by the bicycling contributed to the heat balance of the body. In a large number of arctic and tropical animals previously investigated the shivering and gross muscular activity which took place in the confinement of a metabolic chamber had the same effect. This contrasts with results taken from mice, rabbits, and lemmings which were forced to run on a treadmill in the cold (HART, 1952, 1953; HART and HEROUX, 1955). Here cold and work produced additive effects on the metabolism, *i. e.* in terms of heat economy the work on the treadmill was wasted. Nevertheless, one may hardly from this conclude that muscular exercise cannot substitute for the metabolic response to cold (BURTON and EDHOLM, 1955). The usual experience in animals and man is, of course, that exercise in the cold improves the heat balance and the high efficiency reached in our ergometer experiments rather identifies exercise as one of the basic factors involved.

[1] Wool underwear, shirt, knickers, heavy sweater, wool socks and heavy shoes, trench coat and wool cap, mittens, scarf.

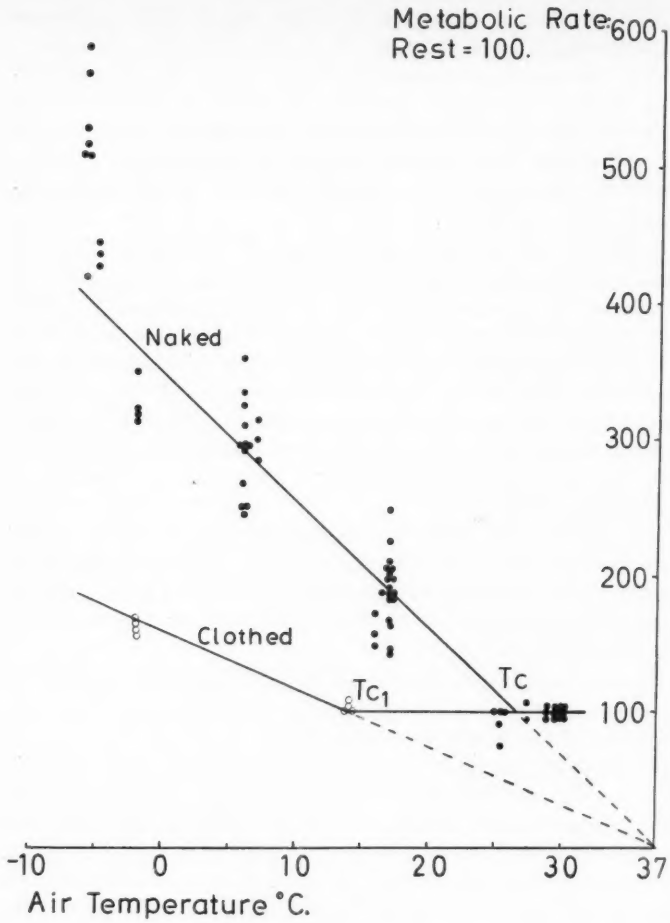

Fig. 1. Metabolic cost of thermoregulation in naked man and in man clothed in ordinary winter sport clothing. Critical temperatures: T_c and T_{c_1}. Diagonal lines conform with Newton's law of cooling.

It is believed that in man as in warm-blooded animals the critical temperature is an important index of climatic adaptation, which would be well worth a comparative study among the various human races.

Summary.

Investigations have been made on the critical temperature in naked man and the metabolic cost of maintaining an adequate heat balance below this point. The experiments were performed on a bicycle ergometer in a cold room, by letting the subjects do just enough work to avoid becoming cold. The critical temperature was found to be approximately 26° C, confirming earlier studies, and below this point the heat production increased approximately in proportion to the body-to-air temperature gradient, conforming with Newton's law of cooling and with data from most other mammals and birds.

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Fibrinolysokinase Activity in Animal and Human Tissue.[1]

By

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Received 22 March 1956.

Plasmin, the proteolytic enzyme in blood, is formed by activation of its precursor *plasminogen*. This activation can be performed by a number of activating agents (for references see ASTRUP 1954, 1956). Animal tissues contain an activator of plasminogen, which is retained in the solid tissue proteins, and can be brought into an aqueous solution only by special treatment such as with potassium thiocyanate (ASTRUP and STAGE 1952). This tissue activator is a fairly thermostable compound especially at acid reaction (ASTRUP and STERNDORFF 1956).

Fibrinolytically active human blood contains a plasminogen activator (MÜLLERTZ 1953). This activator can be formed by activation of a precursor, a proactivator, in blood and the transformation of the proactivator into the plasminogen activator is effected by streptokinase (MÜLLERTZ and LASSEN 1953, MÜLLERTZ 1955). This blood activator differs from the tissue activator. It is easily soluble in water and is very thermolabile especially at acid reaction (MÜLLERTZ 1954, 1955). These properties the blood activator shares with activating agents in a number of other fluids in the organism (for references see ASTRUP 1954, 1956). Because of the possibility that the precursor of the plasminogen activator in blood is normally activated by compounds

[1] Preliminary communication: Conference on Hemorrhagic Diseases. Marquette University School of Medicine, Milwaukee, May 21—22, 1954.

in the organism similar in action to streptokinase a search for such physiological lysokinases has been made in blood (MÜLLERTZ 1954) and tissues (ASTRUP 1954). However, only fairly small activities were encountered. The present study deals with tissue fibrinolysokinase. The interactions concerned are shown diagrammatically in Fig. 1.

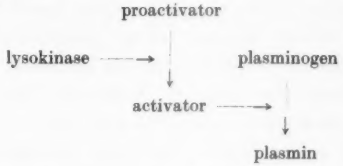

Fig. 1. Scheme representing the effects of fibrinolysokinase and the plasminogen activator formed.

Materials and Methods.

The plasminogen activating effect was estimated on normal fibrin plates (0.1 per cent fibrin) prepared with plasminogen containing bovine fibrinogen (ASTRUP and MÜLLERTZ 1952). Heated plasminogen-free fibrin plates were prepared by heating for 45 mins. at 85° (LASSEN 1952). The activity (average of triplicates) was recorded as the product in sq. mm of two diameters of the lysed zones. Thrombin (bovine) was kindly supplied by the *Løvens kemiske Fabrik*, Copenhagen. Preparations containing proactivator were made from human milk according to ASTRUP and STERNDORFF (1953). Though human blood is a better source of the proactivator, preparations made from human milk contain smaller amounts of inhibitory substances (estimated as antitrypsin) and they are free from plasminogen. The streptokinase ("Varidase") used in control experiments was kindly supplied by the *Lederle Laboratories*. Preparations of bovine plasminogen were made according to the procedure of ASTRUP and STERNDORFF (1953).

Results.

The estimations of the lysokinase activity in tissue extracts are complicated because of the presence of a plasminogen activator in the extracts. When solutions containing increasing concentrations of the extract are applied to the fibrin plates an increase in lytic effect is produced because increasing amounts of plasmin are formed by conversion of the plasminogen contained in the fibrin. If compounds with lysokinase activity are present in the tissue extracts no additional effect is obtained on normal

fibrin plates because the bovine fibrinogen used contains practically no activator precursor. Freedom from contamination with this proactivator can be secured by applying solutions of streptokinase (1 mg in 50 ml) to the fibrin plates. Because the fibrinogen preparations vary to some extent fibrin plates prepared from some batches may give more than a trace of reaction with the streptokinase solution. Such batches have to be discarded in experiments where absence of a proactivator is essential. When the absence of protease activity in the extracts has been secured by applying the solutions to heated fibrin plates, and in addition the freedom from more than a trace of proactivator in the normal plates has been controlled, the activity estimated on these fibrin plates is a measure of the concentration of plasminogen activator in the extracts. If solutions of proactivator (controlled on normal plates for the absence of plasminogen activator) are added to the tissue extracts and the mixtures applied to the normal fibrin plates, no additional activity is obtained if the extract contains only the plasminogen activator. If a compound with lysokinase effect is also present this should react with the added proactivator and form plasminogen activator, which in turn should produce increased activity on the normal fibrin plates. The lysokinase activity of an extract is therefore estimated from the difference in activities obtained in the presence and in the absence of the added proactivator. If the extract contains any appreciable amounts of plasminogen activator the direct effect on the fibrin plates is great and the increase in activity obtained by addition of a proactivator solution has to be fairly large in order to be taken as an indication of lysokinase effect in the tissue extract. In extracts of low activator activity the presence of lysokinase activity is more easily estimated. Unfortunately the tissues in which a lysokinase effect can be expected are usually potent sources of the tissue plasminogen activator. This applies especially to the human organism, where the highest concentrations of compounds with lysokinase effect should be expected in view of the high proactivator concentration in human blood (MÜLLERTZ and LASSEN 1953, MÜLLERTZ 1955), and the high plasminogen activator concentration in spontaneously active human blood (MÜLLERTZ 1953). It appears from what has been said here, that the investigation of lysokinase active compounds in tissue extracts belongs to one of the more complicated problems in the study of fibrinolysis.

Table 1.

Activity of Rabbit Organs (estimated as described in text).

Organ	Protease activity	Activator activity	Activator + lysokinase activity
Lung: supernatant	0	0	0
» sediment	0	127	120
Lymph gland: whole	0	144	161
Adrenal: supernatant	0	0	0
» sediment	0	128	100
Kidney: supernatant	0	182	182
» sediment	0	152	178
Kidney cortex: supernatant	0	134	156
» » sediment	0	169	*210*
» *marrow:* whole	0	187	183

In an experiment with *rabbit* organs the tissues were minced in a Potter homogenizer with 3 vol. of 0.38 per cent sodium citrate. After centrifugation at 18,000 rev. per min. for 5 mins. the supernatant was separated and the sediment resuspended in citrate to the original volume. The resulting supernatant and suspension were tested for protease activity on heated fibrin plates; for activator activity on heated plates after addition of plasminogen (1 ml organ extract + 0.5 ml bovine plasminogen solution (15 mg per ml) + 0.5 ml 0.9 per cent NaCl); and for lysokinase activity on heated plates after addition of plasminogen and proactivator (1 ml organ extract + 0.5 ml bovine plasminogen solution (15 mg per ml) + 0.5 ml human proactivator solution (20 mg per ml)). The following organs were tested: Kidney, kidney cortex, kidney marrow, lung, adrenals and lymph glands. The kidney marrow and lymph glands were not divided into subfractions. The results are presented in Table 1. Apparently only the sediment from the kidney cortex and possibly from the whole kidney show sufficient increase in activity by addition of proactivator to indicate a lysokinase activity in the tissue. In other experiments evidence was also obtained for some activity in the kidney marrow. An extract of pork kidney gave a similar low increase in activity. An experiment with different organs treated with barbital buffer (0.05 M; pH 7.8; containing 0.1 M NaCl) gave less activity in all cases and no lysokinase effect (measured on normal fibrin plates with no excess plasminogen added). Organs from guinea pigs similarly treated with barbital buffer and tested on normal plates with no extra plasminogen added were completely negative. Kidney and lung showed slight activator activity.

Human organs (from two cases of heart death) were disintegrated in 3 vol. of barbital buffer and left for 15 mins. at room temp. After centrifugation (15 mins. at 2,500 rev. per min.) the supernatant was

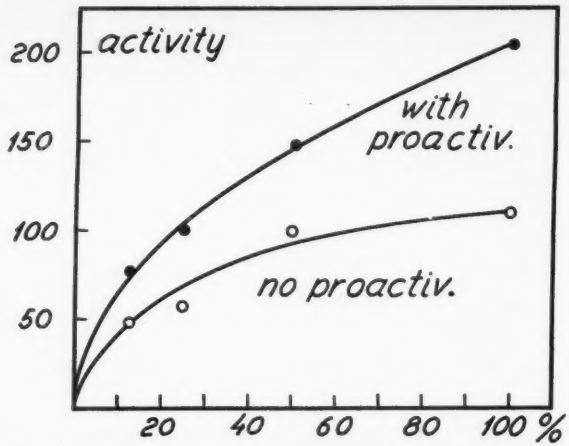

Fig. 2. Effect of addition of proactivator on activator activity of KSCN-extract of human kidney.

Abscissa: Conc. of extract in per cent of final solution.
Ordinate: Activity as diameter product of lysed zones (triplicates).

Test solution: Serial dilutions of extract mixed with equal volumes of barbital buffer or a solution of human proactivator in barbital buffer (20 mg per ml).

separated and the sediment resuspended in barbital buffer to the original volume. No increased effect was found for lung, lymph glands and adrenals. Again the kidney tissue gave a slight increase in the presence of proactivator and the supernatant was the most active.

An acetone-dried preparation was made by disintegrating 70 g human kidney cortex with 15 ml 0.9 per cent NaCl in a Waring blendor. Then 200 ml acetone were added in the blendor and the disintegration continued for a few minutes. After centrifugation the tissue was again treated twice in the blendor with acetone (150 ml and 100 ml). Finally it was stirred with dry ether, filtered, washed with ether and dried in the air. Yield: 10 g.

Of this preparation 1 g was treated for 2 hours with 25 ml barbital buffer and filtered (solution A). Another lot (1 g) was similarly treated with 25 ml 1 M potassium thiocyanate and filtered (solution B). Potassium thiocyanate has been used successfully in the extraction of the plasminogen activator from tissues and does not interfere with the estimation of its activity by the fibrin plate method (ASTRUP and STERNDORFF 1956). The solutions were tested on normal fibrin plates in the presence of proactivator. The barbital extract (solution A) had almost no activator activity and only a trace of lysokinase activity. The activator effect of the KSCN-extract (solution B) was increased by addition of the proactivator solution and therefore apparently contained a compound with lysokinase activity (see Fig. 2).

In additional experiments with untreated human tissues kidney extracts showed variable results and some were completely negative. Between these were cases of heart death, death by shock and by carbon monoxide poisoning. Tissues from lung, liver and adrenals were always negative. In one case (barbituric acid poisoning) the lymph glands showed significant lysokinase activity. More than a 100 per cent increase was obtained in the supernatant as well as in the sediment from the extraction with barbital buffer. In the other cases the high plasminogen activator activity of the lymph glands increased only insignificantly when proactivator solution was added.

Discussion.

As is apparent from the scheme in Fig. 1 the term fibrinolysokinase (or shortened: lysokinase) is used to designate compounds activating the precursor of the plasminogen activator. This is the effect shown by streptokinase and the term has been chosen in order to be able to retain the name of streptokinase for the bacterial compound activating the proactivator. For these reasons the term fibrinokinase previously used for the tissue activator has been abandoned (see ASTRUP 1954). The term lysokinase used by LEWIS, FERGUSON and JACKSON (1949) for compounds of bacterial origin with no effect on bovine plasminogen probably covers activators of the proactivator. It thus corresponds to the above terminology. Because of some discrepancies in the effects of the staphylococcal substance there is some doubt about the term staphylokinase (LEWIS and FERGUSON 1951 a). They have also used the term fibrinolysokinase activators for activating agents from tissues (LEWIS and FERGUSON 1950). These compounds are true plasminogen activators and should be termed accordingly. The activating agent found by LEWIS and FERGUSON (1951 b) in blood was termed serum fibrinolysokinase and assumed to convert plasminogen into plasmin. Experiments by MÜLLERTZ (1954) indicate that this compound exerts its effect by activation of the activator precursor. Consequently it is a true fibrinolysokinase according to the terminology used here.

The search for a compound with fibrinolysokinase activity in animal and human tissues has been only partially successful, as has the search for a similar compound in human blood (MÜLLERTZ 1956). The results of some of the experiments indicate the presence of compounds with lysokinase activity, and in fact it would be rather difficult to explain the observations without

suggesting an effect of a fibrinolysokinase. However, most of the results were negative in this respect. As mentioned before complications arise from the presence in the extracts of high plasminogen activator effects. Kidney tissue gave the most promising results, and this might have some connection with the observation that urine contains large amounts of a plasminogen activator very similar in nature to the labile activator which can be formed in blood (ASTRUP and STERNDORFF 1952). If this compound is not produced by simple excretion of an activator constantly formed in the blood, it could be assumed that an activation of the proactivator would occur in the kidneys for its subsequent release into the urine. In a few cases lymph glands also showed significant lysokinase activity. In all other organs the results were too erratic to warrant a definite conclusion. This was also the case, when the organs were obtained from persons where a high spontaneous activator activity is usually found in the blood post-mortem as in carbon monoxide and barbiturate poisoning (MÜLLERTZ 1952, 1953). The final demonstration of a tissue fibrinolysokinase still awaits the preparation in reproducible manner of potent preparations and its separation from the contaminating plasminogen activator. The experiments presented here show that this is not a simple problem.

This investigation received support from The Josiah Macy Jr. Foundation, New York, and the National Danish Association against Rheumatic Diseases.

Summary.

Animal and human tissues have been investigated for compounds activating the precursor of the plasminogen activator. In a few instances fibrinolysokinase activity was demonstrated in kidney tissue and lymph glands. All other organs investigated showed only activator activity and no lysokinase effect.

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The Effect of 2,4-Dinitrophenol on ATPase Activity in the Particulate and Soluble Fraction of Rat Diaphragm.

By

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Received 27 March 1956.

In a previous paper it was shown that 2,4-DNP inhibits the hexokinase reaction in the surviving rat diaphragm (JERVELL, WALAAS and WALAAS 1956). The effect was most pronounced under anaerobic conditions, and could therefore not exclusively be explained by an uncoupling of oxidative phosphorylations by 2,4-DNP.

The possibility that 2,4-DNP stimulates the dephosphorylation of ATP in the rat diaphragm has therefore to be taken into consideration. Earlier it has been shown that 2,4-DNP stimulates ATPase activity in liver mitochondria (HUNTER 1951, LARDY and WEILMAN 1953), and the observation has been made that 2,4-DNP increased the dephosphorylation of ATP in minced muscle (LARDY and ELVEHJEM 1945). A stimulation of ATPase activity in the mitochondria of pigeon breast muscle has been demonstrated by CHAPPEL and PERRY (1954).

In the present work the stimulating effect of 2,4-DNP on the ATPase activity in the particulate and soluble fraction of rat diaphragm separated by differential centrifugation is investigated. Furthermore, investigations on the mechanism, of this effect by 2,4-DNP are carried out.

2,4-DNP = 2,4-dinitrophenol. ATP = Adenosine triphosphate.

Experimental.

Differential centrifugation. Diaphragms from 15 to 20 adult rats who had fasted overnight were used, and the various fractions were isolated according to the usual procedure (SCHNEIDER and HOGEBOOM 1950). After removal, the diaphragms were kept in ice-cooled Ringer solution, weighed, cut into small pieces and homogenized for 20 minutes in ice-cold 0.25 M sucrose under cooling in an all glass grinder. The nuclear fraction was removed by centrifugation at 600 × g for 10 mins. and the supernatant was centrifuged at 8,000 × g for 20 mins. The particulate fraction thereby sedimenting was washed in 0.25 M sucrose and recentrifuged at 15,000 × g for 20 mins. This was repeated and the washed particles were suspended in a suitable volume of ice cold 0.25 M sucrose. The supernatant from the first sedimentation of the particulate fraction was recentrifuged at 18,000 × g for 30 mins. The residue was discarded and the supernatant was used for experiments with the soluble fraction, all these procedures being carried out at + 1° C, in an "International" refrigerated centrifuge.

Assay for ATPase activity.

0.5 ml particle suspension (usually equivalent to the weight of 200 mg diaphragm) or 0.5 ml soluble fraction (equivalent to the weight of 50 mg diaphragm) was added to the test system. ATP was most often added to give a final concentration of 0.0025 M. In several experiments KF was added in a concentration of 0.01 M to 0.02 M to inhibit adenylate kinase (NOVIKOFF, HECHT, PODBER and RYAN 1952, SIEKEVITZ and POTTER 1953) and phosphatase activity. Tris (hydroxymethyl) aminomethane/HCl was used as a buffer ("Tris") in a final concentration of 0.03 M. Except when otherwise stated pH was 8.2. The total volume of the incubation mixture was 3.0 ml. In most experiments the incubation was carried out for 20 minutes at 30° C in air. The reaction was stopped by the addition of 12 ml ice cold 3 % $HClO_4$ and tests withdrawn for the determination of phosphorus (FISKE and SUBBAROW 1925).

Ba-salt of ATP from "Sigma" chemical company was used and converted to the Na-salt by ion exchange resin (Amberlite 120). The Na-salts of 2,4-DNP and p-nitro-phenol were Merck analar products. The disodium salt of p-nitro-phenyl-phosphate from "Sigma" chemical company was used (degree of purity 70 %).

Results.

The effect of 2,4-DNP on the particulate fraction.

As shown in Fig. 1, 2,4-DNP stimulates the dephosphorylation of ATP in the diaphragm homogenate. This is mainly an effect on the particulate fraction. There is considerable ATPase activity in the particles isolated from the diaphragm (Fig. 2), and the

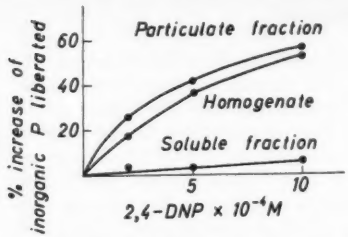

Fig. 1. Stimulation of ATPase activity in different fractions of rat diaphragm by 2,4-DNP.

Incubation mixture (final concentrations): 0.0022 M ATP, 0.01 M $MgCl_2$, 0.02 M KF, 0.03 M "tris"-buffer, pH: 8.2, 0.5 ml of tissue fraction (50 mg diaphragm tissue equivalent). Incubation for 20 mins. at 30° C in 100 % N_2.

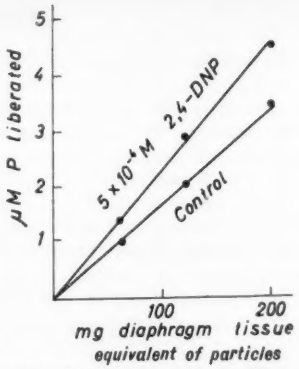

Fig. 2. Stimulation of ATPase activity of particulate fraction of rat diaphragm by 2,4-DNP.

enzyme activity increases in a linear correlation to the amount added. This "ageing" effect observed on the diaphragm particles, can probably be ascribed to the long homogenization period required. As shown in Fig. 3, ATPase activity is stimulated by Ca^{++} and even more so by Mg^{++}. 2,4-DNP stimulated the activity both with and without the addition of metal cations. The effect however was always more pronounced without the addition of metal salts.

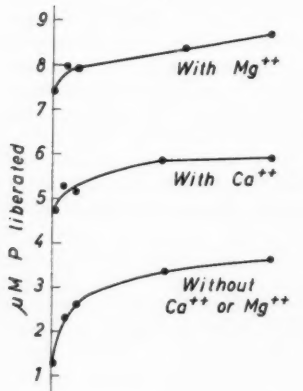

Fig. 3. Effect of 2,4-DNP on ATPase activity of particulate fraction of rat diaphragm when Ca^{++} or Mg^{++} is added. The incubation mixture contained (final concentrations): 0.005 M ATP, 0.005 M $CaCl_2$ or 0.005 M $MgCl_2$ when added.

In Fig. 4 investigations on various concentrations of Mg^{++} and Ca^{++} are reported. A stimulation of ATPase activity by 2,4-DNP generally appeared in these experiments. The highest activity was to be found when ATP/Mg = 1 (molar concentrations) both with and without the addition of 2,4-DNP. With higher concentrations of Mg^{++} inhibition occurred. The activity was not de-

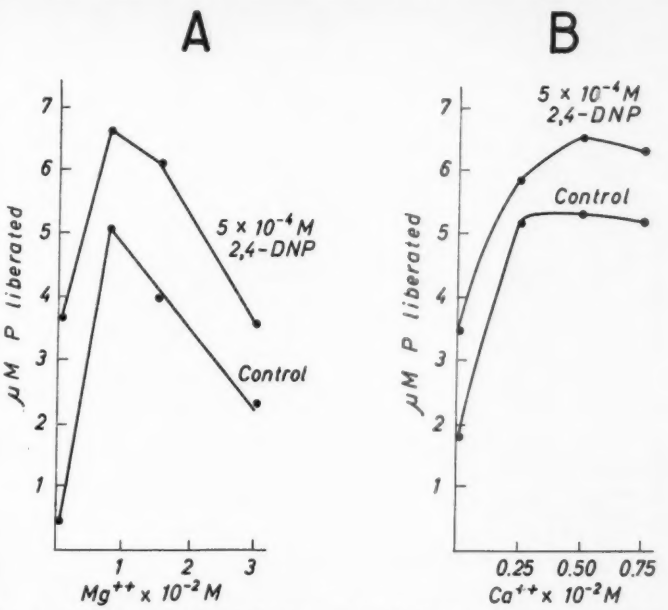

Fig. 4. Stimulation of ATPase activity of rat diaphragm particles by 2,4-DNP at different concentrations of Mg^{++} and Ca^{++}.
A: Different concentrations of Mg^{++}. 0.0075 M ATP was present.
B: Different concentrations of Ca^{++}. 0.0052 M ATP was present.

pendent on the concentration of Ca^{++} to the same degree, although here also the highest activity was found when ATP/Ca = 1.

In "Tris" buffer, optimal enzyme activity was found at pH 9 with a smaller peak at pH 8.5, when Mg^{++} or Ca^{++} were present (Fig. 5). The activation with 2,4-DNP was somewhat different with than without the addition of metals. Without the addition of metal the activation was strongest at lower pH values and the highest activity after the addition of 2,4-DNP was found at pH 7.0. When Mg^{++} or Ca^{++} were added, 2,4-DNP stimulated the activity to the same degree at different values of pH.

The effect of 2,4-DNP on the soluble fraction.

The ATPase activity in the soluble fraction was stimulated by the addition of Ca^{++} and Mg^{++}, but was less sensitive to 2,4-DNP than the particulate fraction (Fig. 6). Mg^{++} produced strong stimulation of the activity, and the maximum activity was found

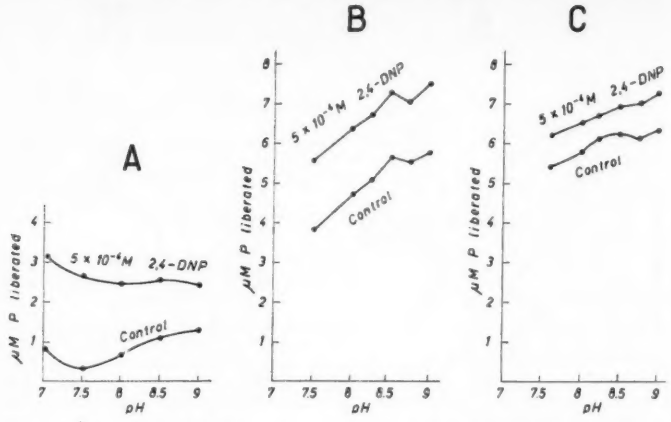

Fig. 5. Stimulation of ATPase activity of the particulate fraction of the diaphragm by 2,4-DNP at different pH.

A: Without Mg^{++} or Ca^{++}.
B: With 0.005 M Mg^{++}.
C: With 0.005 M Ca^{++}.

ATP was added to a final concentration of 0.005 M.

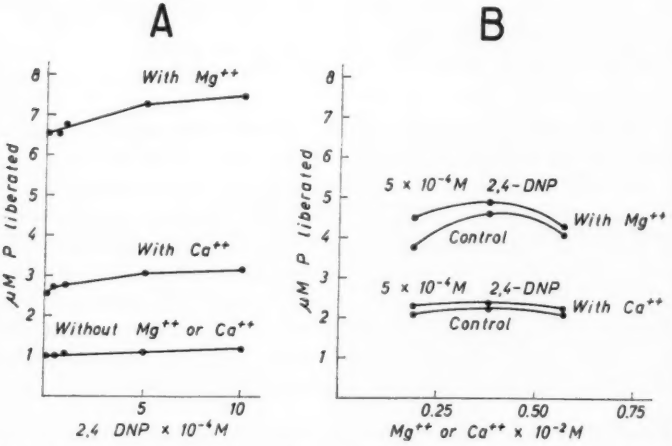

Fig. 6. Stimulation of ATPase activity in the soluble fraction of the rat diaphragm by 2,4-DNP when Mg^{++} or Ca^{++} is added.

A: The incubation mixture contained 0.0037 M ATP, and 0.0037 M $MgCl_2$ or 0.0037 M $CaCl_2$ when added.
B: With 0.0037 M ATP and different concentrations of Mg^{++} or Ca^{++}.

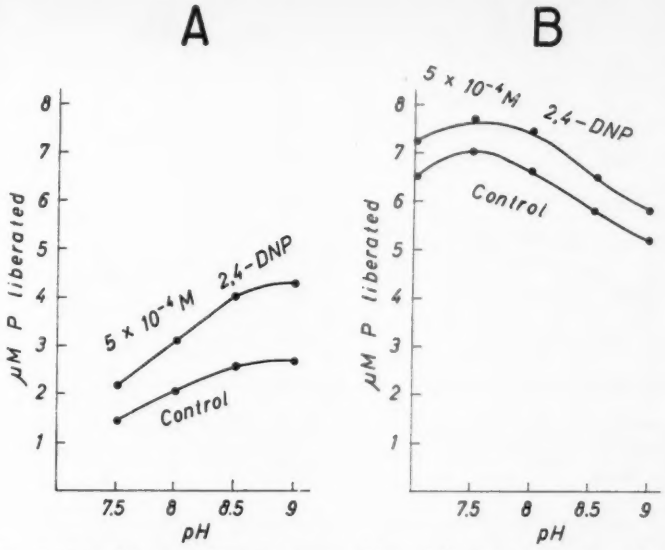

Fig. 7. Stimulation of ATPase activity of the soluble fraction of the rat diaphragm by 2,4-DNP at different pH.
A: Without addition of Mg^{++} or Ca^{++}.
B: With addition of 0.0037 M $MgCl_2$.
The concentration of ATP was 0.0037 M.

at ATP/Mg = 1. 2,4-DNP in a concentration of 5×10^{-4} M produced stimulation of ATPase activity to the extent of 10 % to 60 %. In contrast to the situation in the particulate fraction, the degree of activation was approximately the same with and without the addition of cations.

An optimum of ATPase activity was found in the vicinity of 9.0 (Fig. 7). When Mg^{++} was added however, a shift of the optimum towards lower pH values around 7.5 was found. 2,4-DNP stimulated enzyme activity to the same degree at all pH values.

The mechanism of the effect of 2,4-DNP.

When 2,4-DNP stimulates reactions, which result in an increased dephosphorylation of ATP, several explanations for this effect may exist.

1. 2,4-DNP appears as acceptor in transphosphorylating reactions from ATP.
2. 2,4-DNP affects the dissociation of ATP and thereby the formation of the enzyme substrate complex.

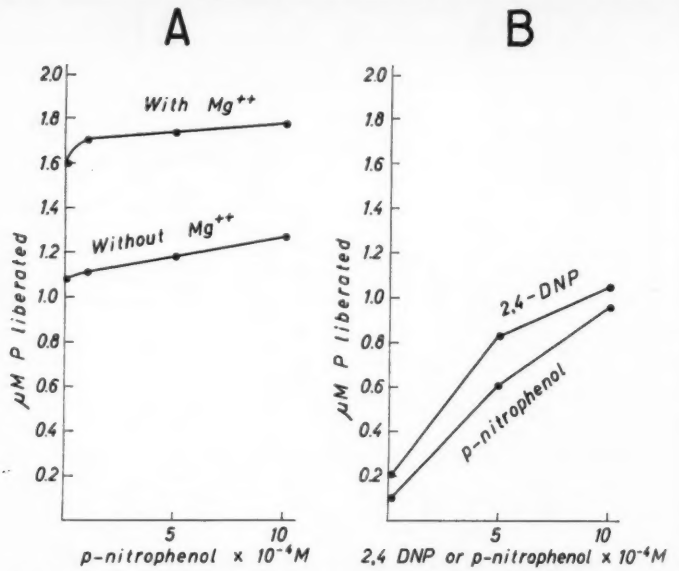

Fig. 8. Stimulation of ATPase activity of particles from the diaphragm and the liver by nitrophenols.

A: Rat diaphragm particles. The incubation medium contained (final concentrations): 0.0023 M ATP, 0.02 M KF, and 0.01 M $MgCl_2$ when added.

B: Rat liver particles. The incubation medium as described in A.

3. 2,4-DNP is bound to enzymes and changes the properties of enzymes which are of importance for the dephosphorylation of ATP.

The first possibility assumes a phosphorylation of 2,4-DNP with a secondary liberation of inorganic phosphate by the effect of phosphatase. This hypothesis was examined experimentally by means of p-nitrophenol instead of 2,4-DNP. On phosphorylation of p-nitrophenol, there occurs a typical shift in the absorption maximum at alkaline pH from 400 mμ to 310 mμ (Bessey and Love 1952).

Since ATPase of particles isolated from the diaphragm were relatively resistant towards p-nitrophenol, liver mitochondria were used instead in these investigations (Fig. 8). In these experiments however, no disappearance of p-nitrophenol or formation of p-nitrophenyl phosphate could be detected[1] by differential spectrophotometry. In these experiments the phosphatase activ

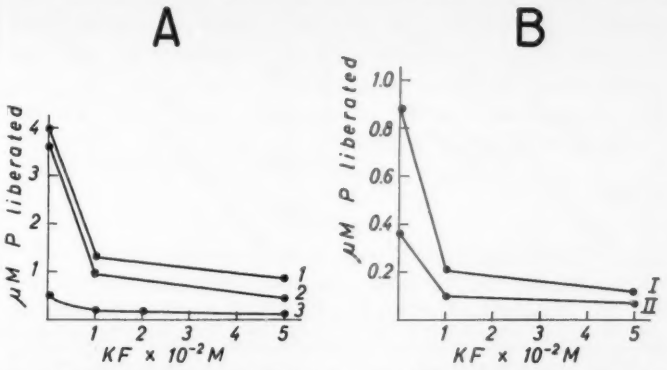

Fig. 9. Suppression of alkaline phosphatase activity of the diaphragm and the liver by KF.

A: Rat diaphragm. 1. Homogenate. 2. Soluble fraction. 3. Particulate fraction. The incubation mixture contained (final concentrations): 0.01 M p-nitrophenyl phosphate, 0.01 M $MgCl_2$, 0.03 M "Tris" buffer, pH: 8.2. Incubation time 60 mins. at 30° C in air.

B: Rat liver particles. The incubation medium contained (final concentrations): I: 0.01 M p-nitrophenyl phosphate. 0.01 M $MgCl_2$. 0.03 M "Tris" buffer pH: 8.2. II: 0.002 M p-nitrophenyl phosphate. 0.002 M $MgCl_2$. 0.03 M "Tris" buffer pH: 8.2 was used.
Incubation was carried out for 60 mins. at 30° C in air.

ity, which is low in particles from diaphragm or liver, was inhibited by 0.02 M KF (Fig. 9). The Mg-activated phosphatase in the soluble fraction of the diaphragm was strongly inhibited by fluoride. The stimulation of ATPase activity by 2,4-DNP was not on the other hand affected by fluoride.

2,4-DNP phosphate and p-nitrophenyl phosphate are relatively acid resistant (Bessey and Love 1952, Lardy and Wellman 1953) and do not liberate inorganic phosphate when subjected to the method of Fiske and SubbaRow. The possibility that unknown labile phosphate esters may occur after the addition of nitrophenols was also investigated in experiments with both the particulate fraction from liver and diaphragm and the soluble diaphragm fraction. Experiments were carried out with and without the addition of Mg^{++} and fluoride. In all cases values for inorganic phosphate according to the methods of Martin and Doty (1949), Lowry and Lopez (1946), Lipmann and Tuttle (1944) were found, identical to the values, according to the method

[1] Nor could there be detected a non enzymatic phosphorylation of 2,4-DNP from ATP by examination in bicarbonate buffer at pH 7.4 without the addition of tissue material, according to a manometric method (Augustinsson 1952).

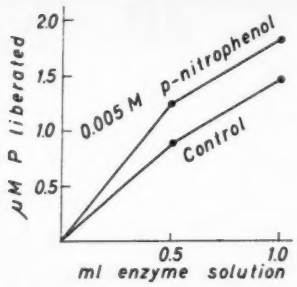

Fig. 10. Stimulation of ATPase activity of acetone desiccated rat liver particles by p-nitrophenol.

90 mg acetone powder was suspended in 10 ml water and dialyzed against distilled water for 24 hours. The residue was centrifuged down and the supernatant used. Incubation mixture (final concentration) 0.0023 M ATP, 0.01 M $MgCl_2$, 0.03 M "Tris" buffer, pH: 8.2. 0.5 ml or 1.0 ml enzyme solution used. Total volume 3.0 ml. Incubation for 20 mins. at 25° C in air.

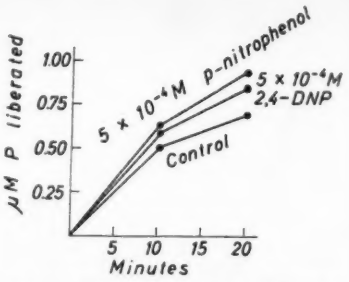

Fig. 11. Effect of 2,4-DNP and p-nitrophenol on 5 times reprecipitated myosin ATPase from rabbit skeletal muscle.

Incubation mixture: 0.2 ml ATP (0.017 M), 0.1 ml $CaCl_2$ (0.1 M), 0.1 ml enzyme solution, 1.0 ml "Tris" buffer (0.03 M), pH: 8.6. Total volume 1.6 ml. Incubation at 10° C in air.

of FISKE and SUBBAROW (1925). So far, these experiments have ruled out the possibility that nitrophenols appear as phosphate acceptors in transphosphorylating reactions with ATP as phosphate donor.

The second possibility, that 2,4-DNP affects the dissociation of ATP, has also been examined experimentally. It is known that Mg^{++} alters pK_4 of ATP (HERS 1952) indicating the formation of Mg-ATP-complex. This was confirmed in our experiments. 2,4-DNP however, did not influence the titration curve of ATP, with or without the addition of Mg^{++}.

In further experiments the third possibility was examined by investigating the effect of 2,4-DNP on partly purified ATPase. Experiments were carried out on acetone powder from rat liver particles and rat muscle particles prepared according to the method of LARDY and WELLMAN (1953). In dialyzed acetone powder from liver particles, p-nitrophenol produced a significant stimulation of ATPase (Fig. 10). Acetone treatment however destroyed nearly all ATPase in muscle particles, and the effect of 2,4-DNP was slight.

An effect of nitrophenols on purified myosin ATPase was

demonstrated as shown in Fig. 11. The activity of myosin ATPase prepared according to BAILEY (1942) was slightly stimulated by 2,4-DNP as well as by p-nitrophenol. As shown recently by GREVILLE and NEEDHAM (1955) and by CHAPPEL and PERRY (1955) a stronger acceleration of ATPase by 2,4-DNP is present at certain ionic strength of the incubation medium.

Discussion.

The present work confirms the suggestion previously made, that 2,4-DNP decreases glucose uptake of the isolated rat diaphragm *primarily* by stimulation of ATPase activity.

Evidence for this assumption is given by the observations made:

1) 2,4-DNP decreases glucose uptake of the surviving diaphragm under anaerobic conditions.
2) 2,4-DNP inhibits hexokinase reaction in the particulate and soluble fraction isolated from the diaphragm.
3) ATPase activity of the particulate and soluble fraction from the diaphragm is accelerated by 2,4-DNP.

This indicates that when 2,4-DNP is added to the diaphragm in vitro, a reduced level of ATP limits the phosphorylating capacity of the hexokinase system. Consequently this may be the reason why 2,4-DNP decreases glucose uptake of the surviving diaphragm.

However, it is still an unsettled question if hexokinase reaction is the rate limiting step for glucose uptake by normal muscle. As indicated in experiments by BELOFF-CHAIN and collaborators (1955), by PARKE, BORNSTEIN and POST (1955) and PARK and JOHNSON (1955), a transport mechanism is involved in the transfer of glucose across the cell membrane into the muscle. The mechanism of this transfer process is not known. The possibility exists that a phosphorylation reaction is involved and an inhibition of the transfer process by 2,4-DNP must also be considered as a possibility.

The particulate fraction which is isolated from the diaphragms exhibits ATPase activity in the same manner as "aged" liver particles. While freshly isolated liver particles do not show ATPase activity (HUNTER 1951, KIELLEY and KIELLEY 1951) various experimental procedures will activate a latent ATPase activity (POTTER, SIEKEVITZ and SIMONSON 1953). The ATPase activity in the diaphragm particles may be ascribed to the procedures necessary for isolation. ATPase in the diaphragm particles is

activated by Ca^{++} and Mg^{++} in the same manner as "aged" liver particles. Under all conditions 2,4-DNP stimulates the activity.

In the soluble fraction of the diaphragm, ATPase activity was stimulated by Ca^{++} and Mg^{++}, and 2,4-DNP accelerated the activity under all experimental conditions. The effect of 2,4-DNP was weaker than the effect on the particulate fraction, but completely reproducible. Thus there are fundamental similarities for the effect of 2,4-DNP on the particulate and soluble fraction of rat diaphragm.

The mechanism by which 2,4-DNP accelerates ATPase activity in the isolated particles is not known. The experiments have excluded the occurrence of a phosphorylation of nitrophenol or a change in the dissociation of ATP.

It has previously been suggested that 2,4-DNP changes enzyme specificity with a shift from transphosphorylase to a dephosphorylating enzyme. The effect by 2,4-DNP demonstrated on soluble muscle systems is in accordance with this hypothesis.

The effect by 2,4-DNP on diaphragm particles was stronger at pH 7 than at pH 9 when no cations were added. This may indicate that the effect depends upon the dissociation of the phenolic group. However, the Mg^{++}-activated and Ca^{++}-activated ATPase was stimulated by 2,4-DNP to the same extent at different pH. The problem of the mechanism requires further studies of the interaction of nitrophenols with purified phosphorylating enzymes.

Summary.

1. The effect of 2,4-DNP on the ATPase activity in the particulate and soluble fraction of the rat diaphragm is investigated.
2. A particulate fraction isolated from the diaphragm by centrifugation at 8,000 × g for 20 mins. showed ATPase activity which was accelerated by Ca^{++} and Mg^{++}. 5×10^{-4} M 2,4-DNP stimulated ATPase activity further, most pronounced without the addition of Ca^{++} and Mg^{++}. Without the addition of metal salts 2,4-DNP accelerated the ATPase activity more strongly at pH 7.0 than at pH 9.0.
3. The ATPase activity in the soluble fraction from the diaphragm obtained by centrifugation at 15,000 × g for 20 mins. was high and activated by Ca^{++} and Mg^{++}. 5×10^{-4} M 2,4-DNP produced a slight stimulation of ATPase activity. Purified myosin ATPase was slightly stimulated by 2,4-DNP and p-nitrophenol.

4. No evidence has been found that nitrophenols are being phosphorylated in these experiments. Nor has it been found that 2,4-DNP affects the dissociation of ATP. Probably the acceleration of ATPase by nitrophenols is due to an alteration of the specificity of phosphorylating enzymes.
5. The experiments indicate that 2,4-DNP decreases glucose uptake of the isolated diaphragm by acceleration of ATPase activity with subsequent inhibition of the transphosphorylating reaction involved.

We wish to express our sincere thanks to Eli Lilly and Company and to the Norwegian Research Council for financial support.

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Species Differences in the Water Taste.

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Received 11 April 1956.

When the existence of specific fibres in the frog responding to water first was discovered (ZOTTERMAN 1949) it was believed that these fibres were peculiar to amphibians and that they might serve a particular purpose in the regulation of the water intake in these animals. From more recent research (LILJESTRAND and ZOTTERMAN 1954, COHEN, HAGIWARA and ZOTTERMAN 1955) it became fully evident that even warmblooded animals possess fibres which respond specifically to the application of water on the tongue. Thus it was found that there was an immediate and slowly subsiding response to the application of water to the tongue in the cat, the dog and the pig. In a previous investigation on the sweet taste (ANDERSSON, LANDGREN, OLSSON and ZOTTERMAN 1950) it was found that there were great differences in the responses to sweet tasting solutions in the cat and in the dog. The study of the water taste has in the present investigation been extended to include the rabbit and the white rat as the activity of the chorda tympani taste fibres of these animals has recently been studied by Pfaffmann (1953, 1955) and by BEIDLER (1953, 1955).

Methods.

Three cats, 4 rabbits, 3 pigs and 8 rats were used in this investigation. All the animals except the pigs were anaesthetized by intramuscular injection of chloralose-urethane solution (0.05 g chloralose and 0.25 g urethane in 7 ml of Ringer's solution per kg body weight). The pigs were narcotized by intravenous injection of nembutal in an ear vein.

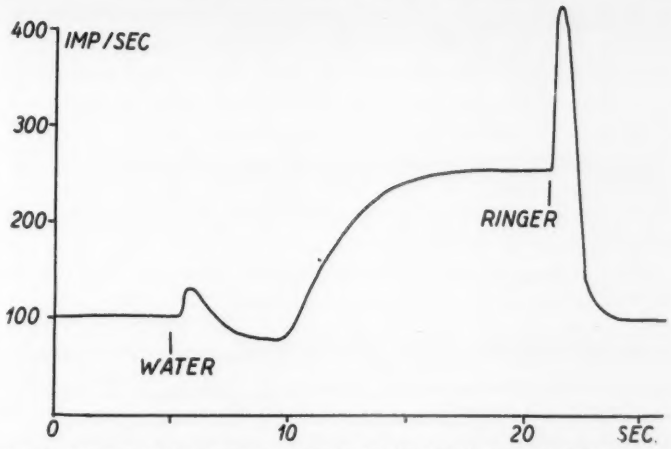

Fig. 1. Diagram of the impulse discharge in the chorda tympani of the rabbit produced by the application of 15 ml of distilled water upon the tongue and a subsequent application of the same amount of Ringer's solution. This graph was constructed by counting spikes in a high speed record from a whole chorda tympani nerve.

After resection of the mandible the chorda tympani was isolated in a manner previously described (ZOTTERMAN 1936). In order to avoid the stimulation of warm and cold fibres running in the chorda tympani (see DODT and ZOTTERMAN 1952) the test solutions were kept in a water bath at 26—28° C. In most experiments on the whole nerve the temperature of the solution at the moment of application was controlled by a thermojunction placed on the surface of the tongue, the deflexions of the galvanometer being recorded simultaneously with the action potentials. When leading off from the entire chorda tympani nerve an integrating device has been used as described in previous papers (GERNANDT, LILJESTRAND and ZOTTERMAN 1946, LILJESTRAND and ZOTTERMAN 1956).

Results.

Rabbit. In the cat and the dog there is a prompt response to water, but pouring water on the tongue of the rabbit produces first a diminution of the spontaneous activity of the taste fibres followed after several seconds by a slow increase in frequency of impulses which then reach a constant fairly high level (see Fig. 1). When, after the impulse frequency has reached this high level, Ringer's solution is poured upon the tongue there follows a sudden phasic response after which the impulse frequency rapidly attains the previous basic value. A subsequent application of

Ringer's solution has no effect. Successive applications of water produce each time a slight immediate diminution of the persistent discharge elicited by the previous water rinse. This immediate diminution of the total response will gradually subside with each application of water. After each rinse there follows a new slow rise of the discharge to a higher level than that produced by the previous application of water until, after three or four water rinses, a maximum of discharge is attained. Further applications of water produce no effects.

After rinsing the tongue with distilled water the response to salty solutions was very greatly enhanced. Thus after a water rinse a 0.002 M NaCl solution produced a quite conspicuous effect. This effect is, however, of very phasic nature, the volley subsiding in a few seconds. Such a phasic volley in response to Ringer was observed in the cat after irrigation of the tongue with water (LILJESTRAND and ZOTTERMAN 1954). It was obvious that a water rinse produced an enhanced sensitivity to weak salt solutions. The application of 10 % saccharose in Ringer's solution generally produces in the rabbit a moderate discharge of impulses while a solution of saccharin in Ringer equally sweet to the human tongue does not produce any response when the tongue was previously rinsed with Ringer. The spontaneous activity of the chorda tympani when the tongue is exposed to the saliva is very small. Generally an almost complete adaptation will be obtained after a 0.1 M NaCl rinse or a Ringer rinse.

Pig. As has been reported previously (LILJESTRAND and ZOTTERMAN 1954) the pig has numerous "water" fibres in its chorda tympani nerve. The response to water is not quite as conspicuous in the pig as in the cat. The responding nerve endings adapt fairly slowly. Fig. 2 shows the integrated response to NaCl solutions of different strengths from 0.5 M NaCl to water after previous rinsing with Ringer's solution. It will be seen that the pig responds much more strongly to a 0.5 M NaCl solution than to water. The salt response is obviously stronger in the pig than in the cat, while the water response, although quite obvious, is somewhat weaker than in the cat. The response to hypotonic NaCl solution comes, however, at a higher concentration than in the cat. Thus it will be seen from Fig. 2 that the pig's chorda tympani gives a quite conspicuous response to 0.05 M NaCl. This response is a true "water" response *i. e.* is built up by "water" fibre activity alone. As the application of Ringer's solution in Fig. 3 shows,

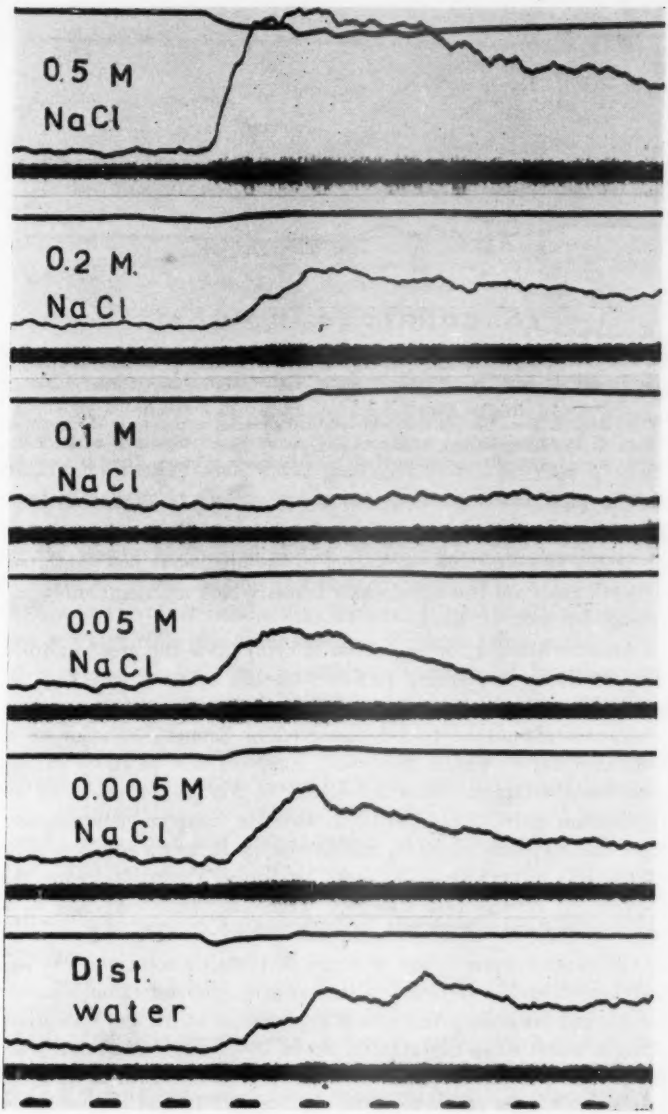

Fig. 2. Records from the chorda tympani of the pig on application of NaCl solutions of different concentrations to the tongue, *A*. 0.5 M. *B*. 0.2 M. *C*. 0.1 M. *D*. 0.05 M. *E*. 0.005 M and *F*. distilled water. In each tracing are recorded from top to bottom: the temperature of the surface of the tongue, the integrated response from the chorda tympani and a direct spike response. Time in seconds.

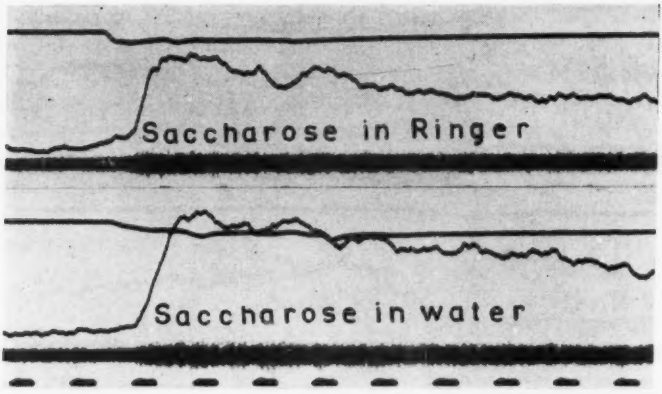

Fig. 3. Records from the pig's chorda tympani showing responses to the application of *A*. 10 % saccharose in Ringer and *B*. 10 % saccharose in water. Both records were taken after previous rinse with Ringer's solution. Time in seconds.

there is no response to Ringer after a previous Ringer rinse. Thus the water fibres of the pig seems to be somewhat less inhibited by NaCl than are the cat's water fibres which are completely inhibited by 0.03 M NaCl.

Characteristic for the pig's chorda tympani is the great number of taste fibres responding to sweet-tasting solution (see Fig. 3). In an animal possessing specific "water" fibres sweet-tasting substances should be tested dissolved in Ringer's solution or a NaCl-solution to which the "salt" receptors can be more or less completely adapted. As will be seen in Fig. 3 the effect of an application of 10 % saccharose in water is conspicuously stronger than the response to 10 % saccharose in Ringer's solution. The tongue was in both cases irrigated by Ringer's solution before the application of the test solution. Thus the total response to a sugar solution in water will be definitely stronger than the response to the same concentration of sugar in Ringer's solution. The initial height of the response will, however, depend upon whether the tongue previously was rinsed by water or by Ringer's solution. After a water rinse the application of 10 % sugar in Ringer's solution will produce a phasic response of "salt" fibres in addition to the sweet fibre response. After rinsing with Ringer's solution the same test solution will stimulate only the sweet fibre endings while sugar in water will stimulate the water fibres, which adapt

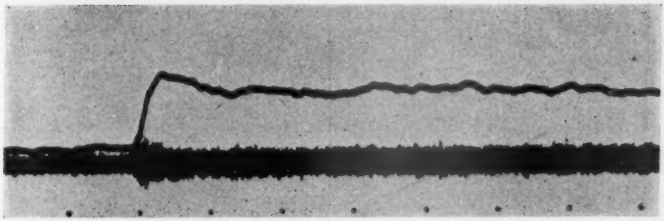

Fig. 4. Records from the rat's chorda tympani nerve showing that after a previous water rinse the application of Ringer's solution produces a sudden and lasting discharge. Time in seconds.

fairly slowly, in addition to the sweet fibres. The phasic response to Ringer's solution and weak NaCl solutions after a water rinse is quite as strong in the pig as in the cat and rabbit.

Rat. In the white rat no positive response to water was obtained. Rinsing the tongue with water produced in the rat only a total cessation or a great diminution in the spontaneous activity of the taste fibres of the chorda tympani. After a while this spontaneous activity slowly reappeared and it attained its original value in a few minutes. While lacking any positive response to water the rat displays on the other hand a very high sensitivity to salt solutions. After a water rinse the application of Ringer's solution upon the tongue produced not only a sudden discharge of impulses, as in the cat, but also a lasting discharge (Fig. 4). This discharge after an application of Ringer's solution underwent in a few minutes a gradual diminution to the level of the previous spontaneous activity. The observation made by BEIDLER (1953) that the rat responds to solutions of NaCl as weak as 0.002 M could be confirmed. Even this low concentration of NaCl applied after a water rinse, produced a continuous response which did not cease but gradually increased during a couple of minutes to attain finally the somewhat higher level of the previous spontaneous activity.

Contrary to the findings of BEIDLER (1953) the rats in this investigation responded fairly promptly and fairly strongly to 0.01—0.02 M quinine solutions. Repeated applications of 0.02 M quinine solution led, however, to a great diminution in the response. The applications of these strong quinine solutions in the rat were, however, followed by a very conspicuous increase in the response of the taste fibres to NaCl solutions as seen in Fig. 5.

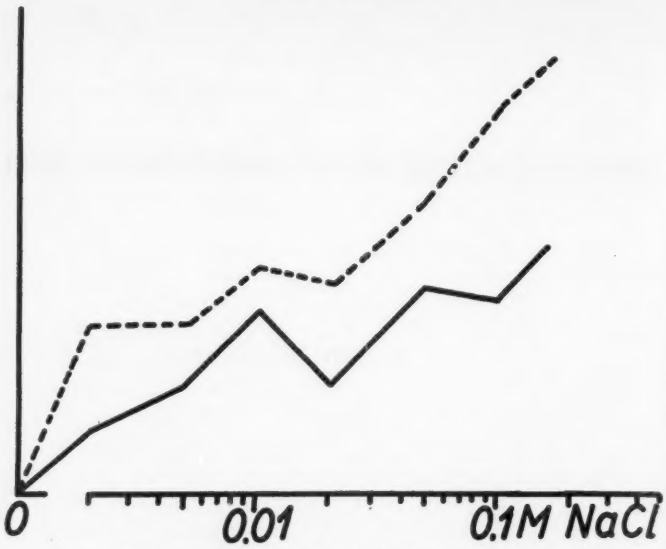

Fig. 5. Graph showing the relative impulse frequency in the chorda tympani of the white rat responding to NaCl solutions of various molarities after a previous water rinse. Solid line before, broken line after the application of 15 ml of 0.02 M quinine hydrochloride solution.

This phenomenon, which was observed in all the rats tested, will be studied in more detail in a following paper.

The rat responded to sweet-tasting solutions but not as strongly as did the pigs. In common with the dog, the pig and the rabbit, all of which respond to saccharose, none of the rats tested in this investigation responded to a saccharine solution, which to the human tongue tasted as sweet as a 10 % saccharose solution.

Discussion.

In recent years PFAFFMANN (1953, 1955), BEIDLER (1953) and BEIDLER, FISHMAN and HARDIMAN (1955) using electrophysiological methods have noticed very great species differences in the responses of the taste fibres of the chorda tympani. Thus both PFAFFMANN and BEIDLER have observed the great difference between the rat and the cat in respect to their response to weak NaCl solutions as well as to quinine. BEIDLER, FISHMAN and HARDIMAN (1955) also noticed that "the spontaneous activity observed

during a water rinse is small in the guinea pig, hamster and dog; moderate in the cat and large in the rabbit. This activity in the rabbit can be suppressed with low concentrations of NaCl." Further they remark that the resting discharge was very small in the rat. They also noticed that the inhibitory effect of NaCl in the rabbit occurred only after an initial and rapid stimulation. This is exactly the phasic response to Ringer's solution or weaker NaCl solutions after a water rinse noticed in the cat, dog and pig by LILJESTRAND and ZOTTERMAN (1954). While the rat does not show any kind of positive response to the application of water upon its tongue, the response to water in the rabbit is characterized by an initial lowering of the spontaneous discharge followed after 4 to 7 seconds by a slow rise of activity which in a few seconds attains a height corresponding to the height of the almost immediate response in the cat. These delayed responses to water and also to NaCl solutions below 0.1 M are very easily seen in the records of BEIDLER, FISHMAN and HARDIMAN (1955, Fig. 1). It will also be seen from their recordings that weak NaCl solutions cause a phasic response until the concentration attains a value above that of Ringer's solution, to which the receptors of the rabbit adapt very rapidly as do the cat's receptors. In the cat, rabbit and dog it is only hypertonic NaCl solutions that produce a lasting discharge of the "salt" fibres.

The question is therefore whether the rabbit possesses any receptors at all for water. One might believe that the delayed response to water is simply an after-effect of the inhibitory action of water, a kind of a release after an inhibition. However, the great height of activity shown in this delayed effect of water in the rabbit (which often attains a value considerably higher than the persistent discharge recorded after an application of 0.2 M NaCl) speaks against such a view. It is more likely that the effect is due to taste endings which are specifically sensitive to water. In view of the quick response of these fibres in the cat, dog and pig as well as in the frog, their endings must be situated very superficially in the mucous membrane of the tongue. Most probably these naked endings penetrate into the mucus layer covering the surface of the tongue. There are reasons for believing that most of the promptly responding taste nerve fibres display such endings. In order to explain the slow response to water in the rabbit it would be easy to suggest that the endings of the fibres responding to water in this animal are situated more deeply

in the mucous membrane than are the salt fibre endings. This would explain the fact that application of water in the rabbit causes an immediate inhibition of the spontaneous activity followed by a slowly rising activity of endings sensitive to water. But there are other possible explanations for the delayed response to water in the rabbit. It could for instance be due to a delayed transport of ions outward from superficial endings.

The high level of spontaneous activity in the rat's chorda tympani is most likely an expression of the high sensitivity of its taste receptors to NaCl. If we assume that the content of chloride ions in the rat's saliva is the same as that of the dog, it would correspond to a concentration of about 0.05 to 0.1 M NaCl. At such a concentration there should be a rather high level of steady discharge in the rat. In the cat, dog, rabbit and pig on the other hand, the receptors adapt quickly to a NaCl solution of this concentration.

The observation that quinine (0.01 M) in the rat produces a very strong and lasting enhancement of the response to NaCl solutions needs, of course, a more thorough investigation. We know from previous investigations that strong acid and strong alkaline solutions suppress temporarily the water response. Such solutions should thus not be used previous to water tests. With quinine we meet the opposite phenomenon, it increases very greatly the sensitivity of the rat's taste fibre endings to NaCl solutions. It is an action very similar to that of menthol on the cold fibre endings (HENSEL and ZOTTERMAN 1951).

While there was no response in the cat to 10 % saccharose in Ringer's solution, the rabbit, rat and pig responded to sugar, the last animal particularly promptly and strongly, thus indicating that the pig possesses a large number of taste fibres responding to sweet-tasting substances. But as was found previously in the dog (ANDERSSON, LANDGREN, OLSSON and ZOTTERMAN 1950) none of these animals displayed a positive response to a saccharin solution which when applied to the human tongue, gave the same sweet sensation as 10 % saccharose solution. It is very difficult to tell why the present tests of saccharin on the tongue of the rat were all negative in contrast to those of BEIDLER (1953) who reports that saccharin gave a very rapid and large response in the rat. It may indicate that there are not only great species differences but also race differences within one species in regard to the different taste reactions. Thus BEIDLER (1953) also reports

that the application of quinine to the rat's tongue elicits a response which is "barely perceptible", while in the present investigation quinine gave a fairly strong and conspicuous response in the rat. It is, however, noteworthy that it was found that the subsequent application of 0.02 M quinine solutions gave a very reduced response.

The very high sensitivity of the rat to weak NaCl solutions found in these experiments is in concordance with the observations by PFAFFMANN (1953) and BEIDLER (1955) and can in itself explain the high discriminating power of these animals. There is as yet no information in regard to salt-discrimination in the cat, dog and pig. If these animals were entirely dependent upon their salt fibres for their discrimination of salty solutions their discrimination should be rather poor. But these species possess water fibres which might serve very well in the discrimination between water and weak salty solutions. It was also suggested in a previous paper (COHEN, HAGIWARA and ZOTTERMAN 1955) that the cat might be able to discriminate between water and salty solution because of the absence of the water fibre activity when salt solutions are applied to the tongue. But even the cat displays a rather high sensitivity to weak salt solutions in contrast to water. Thus even a 0.002 M NaCl solution was in this investigation found to give a quite obvious though brief discharge of impulses but only after a previous water rinse. It is, of course, very difficult to tell whether these short discharges to weak NaCl solution which appear after a water rinse and then adapt to zero within less than a second can play any part in the discrimination mechanism of animals that have water fibres. As concerns our own discrimination in this respect we have previously suggested that the water fibres would provide a further means for the discrimination (LILJESTRAND and ZOTTERMAN 1954). But lacking direct proof of the existence of any water fibres in man it is premature to discuss whether our own discrimination is due to highly sensitive salt fibre endings alone, as in the rat, or to a dual system of salt and water fibres the activity of which is contrasted against each other because of their reciprocal mode of stimulation.

Summary.

1. The white rat lacks taste fibres discharging on the application of water to the tongue but it has "salt" fibres which give

not only a prompt but also a lasting response to the application of NaCl solutions as weak as 0.002 M.

2. The rabbit displays a delayed response to water while the cat and the pig respond very promptly to the application of water upon the tongue.

3. In all these animals (cat, pig and rabbit) the "salt" receptors are almost completely adapted to the saliva and adapt very quickly to the application of Ringer's solution. After a water rinse they all produce a phasic response to even as diluted NaCl solutions as 0.002 M.

4. The high discrimination of salty solutions in the rat must rest upon its very highly sensitive "salt" fibres while the discrimination in the animals that have "water" fibres is suggested to depend upon a dual system of "salt" and "water" fibres which are reciprocally stimulated.

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Reactions of Hedgehogs, Hibernating and Non-hibernating, to the Inhalation of Oxygen, Carbon Dioxide and Nitrogen.

By

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Received 12 April 1956.

The introduction of controlled hypothermia in cardiac surgery posed many fundamental questions as to the adaptive mechanisms of the warmblooded organism exposed to low temperatures. In this respect, studies of the behaviour of hibernators might give important information. Some experimental studies on hedgehogs were carried out in our laboratory.

In a previous communication (BIÖRCK and JOHANSSON 1955) we reported on temperature effects on the electrocardiogram. In the present paper we report on a comparison of the reactions of hedgehogs, hibernating and non-hibernating, following inhalation of various gas-mixtures. The investigation of these problems was prompted by the paper by LYMAN (1951), and the suggestion of SARAJAS (1954) that discrepancies between changes in body temperature and heart rate might be due to variations in the carbon dioxide content of the blood.

The amazing tolerance of hibernating animals to anoxia has long attracted the attention of scientists. Thus, SPALLANZANI (1803) reported that hibernating marmots tolerated exposure to CO_2 for four hours, whereas a rat and a bird rapidly succumbed. Bats could survive in nitrogen for two hours, an experience that was confirmed by KOENINCK (1899). SAISSY (1815) observed that hedgehogs, bats and marmots could tolerate complete anoxia for one hour. CARLISLE (1805) kept a hibernating hedgehog for half an hour under water with no untoward effects. This experiment

was confirmed by BARKOW (1904), and HALL (1832) found that this treatment, which was tolerated by a hibernating hedgehog for 22.5 minutes, killed a non-hibernating hedgehog after three minutes. Some doubts (VALENTIN 1857) have, however, been expressed as to these experiments. We, therefore, decided to repeat them under conditions, where temperature, breathing rate and electrocardiograms could be recorded.

Methods and Material.

Hedgehogs (Erinaceus europaeus) were used as experimental animals. The studies were performed in a cylindric, air-tight glass-container. A continuous flow of gas could pass through the container by means of two tubes. The inflow was about 2—3 cm above the floor of the container. By means of built-in-wires the animal could be connected to an electrocardiograph.

Pure nitrogen, pure oxygen and various concentrations of carbon dioxide in oxygen were administered. The latter mixtures were determined within a range of $\pm$ 0.2 % by means of a Liston-Becker photoelectric apparatus. During the experiments with the carbon dioxide mixtures the animals were brought directly from the mixture with the lower carbon dioxide concentration to the higher one. Data on the duration of the experiment given later refer to the moment when the gas flow into the container began. The gas flow was sufficient enough to have the container washed out in less than five minutes.

The electrocardiograms were recorded as described previously (BIÖRCK and JOHANSSON 1955). On the application of electrodes the animals often reacted with signs of arousal. If so, the animal was left to re-enter the hibernating state. All animals were observed with regard to respiratory and heart rate at least one hour before the experiment. At any sign of arousal, the experiment was postponed. Respiratory rate was measured for 20—30 minutes, when there was Cheyne-Stokes' breathing. If there was regular breathing, it was measured during five minutes. The temperature was measured in centigrades by means of a thermocouple as previously described (BIÖRCK and JOHANSSON 1955).

The studies on hibernating hedgehogs were performed from late February to early March, at the peak of the winter. The studies on non-hibernating hedgehogs were performed from late June to early July, in pleasant summer climate.

Results.

Hibernating hedgehogs (winter experiments).

Pure *oxygen* was given to two animals with Cheyne-Stokes' breathing. The experiment lasted for 120 minutes. There was no

change in skin temperature (2° C and 6.5° C) during the experiment. There was no noticeable difference in heart rate in the periods of apnea or of normal breathing. — The respiratory rate showed a slight decrease and the Cheyne-Stokes' type of respiration continued.

Pure *nitrogen* was given to six animals for periods of 50 to 120 minutes. There was no change in skin temperatures (2° C—7° C). *The respiratory rate* decreased in all animals from an average of 13/min. to 0—1/min. after 50—120 min. (fig. 1). In two instances there was a slight initial increase from 6/min. to 7/min. and from 12/min. to 17/min., respectively. The decrease in respiratory rate was more rapid at the beginning of the period. Ultimately, a breathing rate of zero was observed in two animals after 120 min. (Both animals were observed for over five min.) In one animal, Cheyne-Stokes' breathing existed prior to the experiment, and persisted, although somewhat less pronounced, until the animal stopped breathing after 70 min. All animals survived.

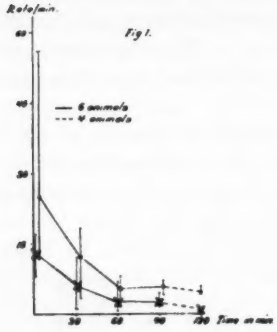
The *heart rate* (fig. 1) increased initially in two cases from 12 to 20 beats/min. and from 14 to 15 beats/min., respectively. This increase lasted for 25 and 15 minutes. In all the other animals the heart rate decreased, and more rapidly at the beginning.

The *electrocardiogram.* In only one animal could P—R be examined. It increased from 0.25 sec. to 0.50 sec. after 115 min. QRS showed a continuous broadening, increasing from 0.23 sec. to 0.25 sec. after 30 min., 0.34 sec. after 60 min., 0.40 sec. after 90 min. and 0.57 sec. after 120 min. — Q T was difficult to evaluate but no great changes were observed. — The QRS_{II}-amplitude decreased from approximately 1.0 to 0.8 mV after 30 min., to 0.4 mV after 90 min. and to 0.1 in one animal after 120 min. There were two exceptions, however, and in one of these there was an increase from 0.7 mV to 1.2 mV after 20 min. This animal was found dead in his cage next morning.

On the whole, the respiratory and heart rate of all the animals as well as the electrocardiograms had returned to previous levels 2—3 hours after the end of the experiment.[1]

A *mixture of 3 % carbon dioxide in 97 % oxygen* was given to 8 animals for 75—120 min. There was no change in the skin temperature, which ranged from 3° to 10.5° C.

[1] Two bats — at a temperature of about 10° C — put into pure nitrogen for 35 and 55 minutes respectively, showed nodal rhythm, decrease of heart rate and QRS amplitude, prolongation of QRS duration and Q—T interval.


Rate/min.
Fig 1.
6 animals
4 animals
Time in min

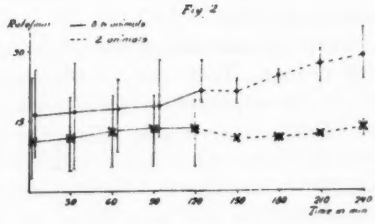


Fig. 2
Rate/min
6 animals
2 animals
Time in min

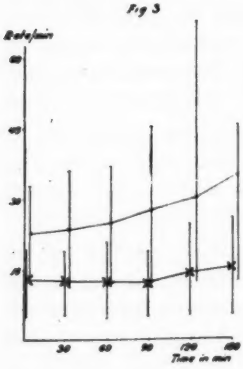


Fig 3
Rate/min
Time in min

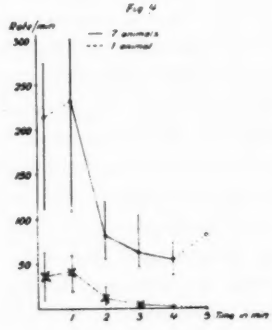


Fig 4
Rate/min
7 animals
1 animal
Time in min

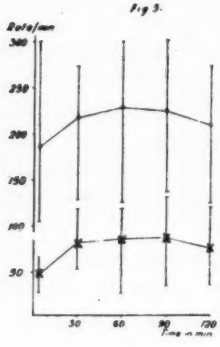


Fig 5.
Rate/min
Time in min

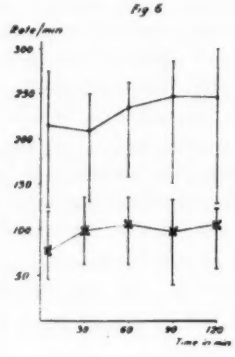


Fig 6
Rate/min
Time in min

The respiratory rate was unchanged in five animals, whereas a slight increase was noted in three instances. Four animals had a Cheyne-Stokes' breathing type. This disappeared in three cases after 15—45 min. In one animal it persisted for more than 105 min. There was no noticeable change in the heart rate or in the ECG.

A *mixture of 6 % carbon dioxide in 94 % oxygen* was given to 8 animals for 70—240 min. There was no change in temperature.

The respiratory rate (fig. 2) increased in 4 animals, decreased in 3 and was unchanged in one animal. It seemed as if the decrease in rate was paralleled by an increased range of inspiratory movements. The Cheyne-Stokes' breathing type in 3 animals disappeared within 45 min.

The heart rate (fig. 2) increased in 6 animals, decreased in one and was unchanged in one. The P—R interval of the electrocardiogram was unchanged in 4 cases, where it could be identified. QRS was, likewise, unchanged except where there was an increase in the heart rate. Q—T did not change. The QRS_{II}-amplitude was unchanged in four animals, but increased in the others, from 0.6 mV to 0.9 mV after 120 min. (4 animals) and to 1.1 mV after 240 min. (2 animals).

Finally, a *mixture of 9.5 % carbon dioxide in 90.5 % oxygen* was given to 8 animals for 120—200 min. There was no change in skin temperature.

The respiratory rate (fig. 3) decreased slightly at first, only to increase again later. There was no instance of Cheyne-Stokes' respiration.

The heart rate (fig. 3) increased somewhat in all the animals. In the electrocardiogram, P—R and Q—T did not show any noticeable changes, QRS was shortened in five cases, from 0.16

Fig. 1. Changes in heart and respiratory rate in hibernating hedgehogs in pure N_2.
Fig. 2. Changes in heart and respiratory rate in hibernating hedgehogs in 6 % CO_2.
Fig. 3. Changes in heart and respiratory rate in hibernating hedgehogs in 9.5 % CO_2.
Fig. 4. Changes in heart and respiratory rate in non-hibernating hedgehogs in pure N_2.
Fig. 5. Changes in heart and respiratory rate in non-hibernating hedgehogs in 6 % CO_2.
Fig. 6. Changes in heart and respiratory rate in non-hibernating hedgehogs in 9.5 % CO_2.

○ — — — — — — — — ○ heart rate
× — — — — — — — — × respiratory rate

sec. to 0.13 sec. after 120 min. In three animals, there was no change. Neither was there a consistent change in the amplitude of QRS; in four instances it was not changed, in another four it increased from 1.0 mV to 1.2 mV after 120 min.

Non-hibernating hedgehogs (summer experiments).

Pure nitrogen was given to seven animals for 3 to 5 min. The *skin temperature* was between 32.5° and 34.5° and did not change during the experiment.

The *respiratory rate* (fig. 4) decreased, after a brief initial increase in 5 animals, from 36/min. to 5/min. after 3 minutes, and to zero after 5 minutes in one animal. The *heart rate* (fig. 4) averaged 214 beats/min. at the beginning of the experiment. In 4 animals there was an initial increase; thus, after 1 minute the rate was 231 beats/min. After 3 minutes, however, the rate was 64/min., and after 5 minutes 83/min. (in one animal).

The *electrocardiogram* showed a P—R of 0.07 sec. prior to the exposure, and this value remained unchanged until a nodal rhythm took over after 1 to 3 min. There was no definite change in the QRS duration. The T wave was found to move towards the QRS complex and finally to be fused with it to a common complex with a duration of 0.07 sec. The amplitude of the QRS_{II} decreased from 0.8 mV to 0.5 mV after 3 minutes.

During the exposure to pure nitrogen the animals often showed vomiting, defecation and muscle-twitching sometimes progressing to cramps. After cessation of the exposure the animals soon regained their previous state: after 2 to 10 minutes the respiratory rate was the same as before the experiment, and also the heart rate and the electrocardiogram returned to normal.

Immediately after the end of the anoxic period some arrhythmias were noticed in the electrocardiogram, such as complete heart block and ventricular premature beats, high S—T junction or negative T_3. One animal died after exposure to nitrogen for three minutes.

A *mixture of 3 % carbon dioxide in 97 % oxygen* was given to seven animals for 60 minutes. The skin temperature remained constant also in this and all the other experiments with CO_2.

The respiratory rate was sometimes difficult to determine, as the non-hibernating animals got disturbed very easily and then developed a rapid and superficial breathing. — Four animals showed an increase, from an average of 27/min. to 48/min. after

60 min., whereas three animals had a decrease from 69/min. to 53/min., sometimes concomitant with a "deeper" respiratory type.

The heart rate decreased in five cases from 227 beats/min. to 136 beats/min. at the end of the experiment. In one case the heart rate remained unchanged at 300 beats/min. and in one animal it rose from 231 to 250 beats/min. There were no other changes in the electrocardiograms.

A *mixture of 6 % carbon dioxide in 94 % oxygen* was given to 10 animals for 120 min.

The respiratory rate (fig. 5) rose in 8 animals and decreased slightly in 2. The average figures were: 47/min. at the onset, 85/min. after 60 min. and 75/min. after 120 min.

The heart rate (fig. 5) increased in 7 animals, was unchanged in 1 and decreased in 2. The average values were: 187 beats/min. at the onset, 229 beats/min. after 60 min. and 208 beats/min. after 120 min. There were no noticeable changes in the electrocardiogram, although the QRS_{II}-amplitude increased in five animals and decreased in three. It was not possible to find any parallelism between these changes in QRS_{II}-amplitude and the changes in respiratory or heart rate.

Finally, a *mixture of 9.5 % carbon dioxide in 90.5 % oxygen* was given to 10 animals for 120 min. The respiratory rate (fig. 6) was at the onset of the experiment on an average 78/min. and rose in all animals the mean being 104/min. after 60 min. and 105/min. after 120 min.

The heart rate (fig. 6) increased in five cases, decreased in four and was unchanged in one animal. The electrocardiogram did not show any changes of P—R or the QRS duration. Q—T increased in seven animals, decreased in one and was unchanged in two. The QRS_{II}-amplitude, likewise, showed increase in five instances, decreased in two and was unchanged in three cases. On an average, there was an increase from 0.7 mV to 0.8 mV.

In order to examine, whether the respiratory rate of hedgehogs subjected to inhalation of CO_2-mixtures increased in the same proportions as in other animals, we performed similar experiments on 7 guinea-pigs. A comparison of the values obtained in these animals with those from summer and winter hedgehogs is given in fig. 7, and indicates, that the general increase in respiratory rate in guinea-pigs is somewhat less marked compared with non-hibernating hedgehogs and somewhat more marked in comparison with hibernating hedgehogs, while the immediate reaction in re-

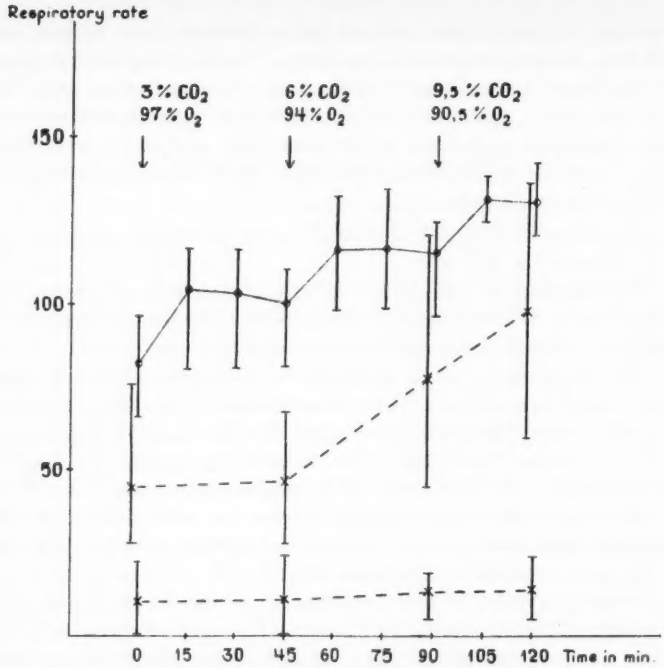


Fig. 7. Respiratory rate in guinea-pigs (————) and in hedgehogs (— — — —) when exposed to different concentrations of carbon dioxide in oxygen. — The middle curve refers to non-hibernating hedgehogs, and the lower curve to hibernating ones.

spiratory rate to the 3 % CO_2-concentration was more pronounced in the guinea-pigs. However, at concentrations of this order, changes in respiratory depth often play a greater rôle, whereas at concentrations above this level, respiratory rate gains in importance. The latter factor, however, is the only one that can be easily measured quantitatively in this kind of experiment.

Discussion.

It is obvious that hibernating hedgehogs are able to tolerate complete anoxia for at least two hours without permanent damage. This period is 20 to 40 times longer than the one tolerated by non-hibernating hedgehogs, and indicates that the aerobic metabolism of the hibernating animal amounts to (or may for a con-

siderable length of time be reduced to) only a small percentage of that of the non-hibernating animal. Whether it is justified to apply the concept of "total" metabolism to the tolerance of anoxia, or whether it must be assumed that there are areas of different metabolic activity, will be discussed later.

During this prolonged exposure to oxygen lack, in pure nitrogen, the heart rate, which was already low, decreased further to $^1/_4$ of its initial value and respiration finally ceased completely. It is interesting that BIGELOW and MCBIRNIE (1953) could interrupt the circulation for one to two hours in groundhogs, cooled down to between 2.5° and 5° C. A comparison of the reactions to nitrogen anoxia in hibernating and non-hibernating hedgehogs displays in both instances a decrease of the heart and respiratory rate. In the non-hibernating animals this was often preceded by a rise which was less conspicuous in the hibernating ones.

When an animal is put into a container, filled with pure nitrogen, the alveolar oxygen store disappears partly by being used up in the lungs and partly by mixing with the nitrogen of the surroundings. The rate of the latter disappearance depends on the ventilatory activity. Although respiratory rate alone may be misleading, it is nevertheless remarkable that the hedgehog can tolerate a much greater number of respirations in nitrogen during winter than during summer — 510 compared with 81 — as can be seen from figs. 1 and 4. The fact that hibernating hedgehogs tolerate a nitrogen atmosphere for a very prolonged period can, therefore, not be explained on the basis of a very slow washing-out of their alveolar oxygen reserve. The metabolism of the hibernating hedgehogs may amount to 1/10—1/20 of that of non-hibernating hedgehogs, in accordance with data from marmots (BENEDICT and LEE 1938) and hamsters (KAYSER 1954). In our series, the mean duration of survival in pure nitrogen was at least 28 times greater for hibernating as compared to non-hibernating hedgehogs. This figure may point to factors of adaptation besides the depressed metabolism itself. Such factors may be stores of oxygen in the body, including erythrocytes pooled in the spleen and physically dissolved oxygen, and possibilities of increased anaerobic metabolism. LIDICKER and DAVIS (1955) have shown that in bats the spleen is enlarged by 500 per cent in hibernation, but is rapidly emptied of stored erythrocytes to the circulation, as the bat becomes active. The spleen is enlarged also in hibernating hedgehogs, but we have not had occasion to ob-

serve its size during experiments with anoxia. However, in two hibernating hedgehogs exposed to an atmosphere of cyanide or carbon monoxide for $1^1/_2$—2 hours, the spleen was not reduced in size. The observation, that hibernating hedgehogs survived in cyanide for about the same length of time as in pure nitrogen, may indicate that anaerobic pathways are able to carry a substantial part of the metabolism, if sufficient oxygen is not available.

Comparing the redox potentials of different organs from hibernating and non-hibernating hedgehogs and the ability of different substances to take an animal out of the hibernating state KLAR (1941), arrived at the conclusion that there is probably a blocking of the aerobic breathing during hibernation while the anaerobic one is undisturbed.

The electrocardiographic recordings from hibernating animals rarely show a typical P wave, but in one instance when this was the case, P—R was observed to become prolonged when the animal was exposed to pure nitrogen. In the non-hibernating animals nodal rhythm replaced the previous sinus rhythm. The QRS duration did not change in the non-hibernating animals, but increased widely in the hibernators. Q—T on the other hand did not seem to change in the winter experiments — except in one case where QRS was very much broadened — but became reduced in the summer experiments. In both series there was a decrease in the QRS_{II}-amplitude in most animals. The observed changes in QRS and Q—T in the hibernators apparently were not only secondary to the changes in heart rate. The QRS duration in the nitrogen experiments considerably exceeded what would be expected from observations at similar heart rates in deep ordinary hibernation. Instead, the QRS complex resembles true anoxic changes, such as are observed as a late event in dying patients. The diminution of the QRS-amplitude may also be an expression of anoxia, as suggested by RANDALL (1944).

The experiments with various concentrations of carbon dioxide show less dramatic reactions. The lowest CO_2-mixture (3 %) was well tolerated by both groups. This mixture was apparently a limit value for respiratory rate at least for some hedgehogs. LYMAN (1951) in hibernating hamsters found a limit value of 2.5 % and KAYSER (1953) reports an increase of oxygen consumption in hibernating animals at a CO_2-concentration above 1.5 %. The decrease in heart rate in the summer hedgehogs was

probably due to a return to more normal values after an initial uneasiness caused by the experimental conditions.

The 6 % mixture induced an increase of breathing and heart rate in both groups, which was also the case with the 9.5 % mixture. It seemed as if there was a greater increase of heart rate in the winter series, whereas in the summer series the respiratory rate increased more. The various and not uniform changes observed in the electrocardiograms do not appear to have any primary significance. The range of carbon dioxide concentration used by us is approximately the same as that used by LYMAN (1951) in his studies on hibernating hamsters and ground squirrels. Our results with hedgehogs differ somewhat from his observations; they indicate a somewhat lower intensity of response of respiration, especially at the high CO_2-concentration and, possibly, a greater influence on the heart than is found in his series. Generally speaking, however, they confirm his contention that hibernators maintain their capacity for homeostatic regulations with regard to carbon dioxide also while hibernating.

It is well known that hibernators are sensitive to changes in the temperature of the surroundings. For these reasons it must be assumed that some parts of the vegetative system in hibernators are kept constantly active. If the metabolic rate of an hibernator is kept at some few per cent of the metabolism in the non-hibernating state, it is possible that these active vegetative mechanisms consume more than a proportional fraction of the available oxygen. The problem of the volume of this oxygen consumption and of methods to safe-guard it would be a rewarding field for future investigations.

The heart rate of hibernating hedgehogs displays rather wide variations at one and the same temperature (SARAJAS 1954, BIÖRCK and JOHANSSON 1955). SARAJAS has suggested that this might at least partly be due to fluctuations in the carbon dioxide content of the blood, connected with the Cheyne-Stokes' respiration. Although our experiments have confirmed also for the hedgehog the sensitivity to carbon dioxide, demonstrated by LYMAN (1951), our experiments with the breathing of pure oxygen — or, in other experiments, air — indicate that SARAJAS' explanation may not be sufficient. In our experiments, in spite of Cheyne-Stokes' breathing, the heart rate remained essentially unchanged both during the apnea and during the period of forced respiration. It may be that in the hedgehog like in the marmot the best

correlation is found between heart rate and heart production (BENEDICT and LEE 1938).

Summary.

Hibernating and non-hibernating hedgehogs were exposed to pure nitrogen and various mixtures of carbon dioxide and oxygen, respectively. Temperature, respiratory rate, heart rate and electrocardiographic details were examined.

Anoxia was tolerated by the hibernating hedgehogs for one to two hours, by the non-hibernating animals for three to five minutes. Hibernating hedgehogs reacted upon prolonged anoxia with a decrease in heart and respiratory rate. P—R and QRS duration increased, while Q—T was unchanged. In non-hibernating animals respiratory and heart rates rapidly decreased. P—R and QRS did not change, while Q—T decreased. In both groups the QRS-amplitude decreased.

Inhalation of 3 % carbon dioxide in oxygen resulted in no significant increase in respiratory rate, whereas 6 % and 9.5 % carbon dioxide provoked an increase in respiratory and heart rate with no consistent changes of the electrocardiogram, in both groups. The increase in respiratory rate in non-hibernating animals was somewhat greater than in guinea-pigs, and in hibernating ones somewhat lower.

This study was supported by a grant from the Medical Research Fund of the Swedish Life Insurance Companies.

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The Rate of Elimination of Catechol Amines in Lymph.

By

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Received 14 April 1956.

Much interest has in recent years been concentrated on the problem of the elimination of adrenaline and noradrenaline under physiological conditions. The present study was made in order to find out to what extent the sympathetic hormones are eliminated by the lymph, which can be considered to reflect the characteristics of the extracellular fluid. As regards another 'biogenic amine', histamine, it is known that lymph contains a specific destructive enzyme, histaminase, in fairly high concentrations (see CARLSTEN 1950), and similar conditions may also apply for catechol amines, though it is also quite possible that their elimination is strictly limited to the tissue cells.

Methods.

Operative procedures: Lymph was collected from 18 cats weighing 3.1—3.8 kg and prepared under chloralose anesthesia (about 75 mg/kg). Body temperature was kept at 38° C, and as a rule arterial blood pressure was recorded. A tracheal cannula was inserted and artificial respiration applied throughout the experiment. Lymph was collected from the thoracic duct. The third and fourth ribs on the left side were cut and retracted cranially. The thoracic duct was tied about 3 cm distally to its entrance into the left jugular and subclavian veins. A glass cannula was inserted into the peripheral end of the thoracic duct. The spontaneously emerging lymph was then led via a short polyethylene

catheter to glass tubes resting on ice and containing some heparine. If the samples were not used at once for assays they were put in a freezing box in order to preserve the activity of any possible enzymes. In five of the experiments adrenalectomy was performed, 1, 2, 3 and 24 hours respectively, prior to the collecting of the lymph. This adrenalectomy was carried out under aseptic conditions. Finally, in one case, lymph was collected from man by cannulating the thoracic duct during an operation on the neck on a patient with cancer laryngis.

Procedures for incubation and biological assays: Quantitative assays were performed on the following samples:

A. Heated and non-heated lymph, before and after incubation.

B. Heated and non-heated lymph with adrenaline or noradrenaline added. Incubated and non-incubated samples.

C. Tyrode solution with adrenaline and noradrenaline added. Incubated and non-incubated samples.

The heating procedure was performed either by boiling the lymph or by heating it at 56° C for two hours. By such procedures all enzymes of a protein nature, possibly contained in the lymph, are destroyed. Before the addition of catechols all samples were kept on ice. The concentrations of added catechols were varied. The standard concentrations used were 1, 5 and 10 μg per ml, but lower and higher concentrations were also studied. The pH of the various samples, as determined by a pH-meter, was generally kept within the range 7.3 to 7.6. A small number of samples with pH-values outside these limits were discarded.

The various samples were incubated in a water bath at 38° C for $^1/_2$, 1. 2 and 4 hours respectively, after which the samples were put on ice for immediate further determinations.

Analysis of the remaining amounts of adrenaline and noradrenaline in the various samples was performed on a piece of the proximal rabbit jejunum, arranged in the ordinary way in a 50 ml capacity bath filled with oxygenated Tyrode solution. The temperature was kept at 38° C and pH at 7.5. 0.2 ml of the sample to be assayed was aspirated in a 1 ml syringe, filled up with Tyrode solution directly from the bath before injection. The injection rate of each sample, as well as the recovery period of the gut between each test, was kept constant. Fresh solutions of adrenaline and noradrenaline were prepared at concentrations to give 0.2, 1 and 2 μg of active substance respectively in 0.2 ml of the various solutions.

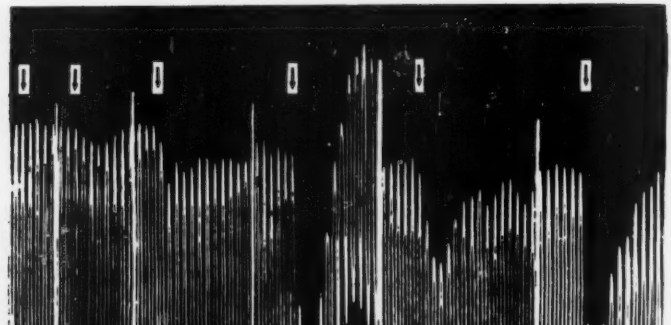

Fig. 1. Inhibitory action on isolated rabbit intestine of:
A: 0.2 ml non-heated pure lymph;
B: 0.2 ml heated pure lymph;
C: 0.2 ml Tyrode solution, incubated for 2 hours with adrenaline, 5 μg/ml;
D: 0.2 ml non-heated lymph, incubated for 2 hours with adrenaline, 5 μg/ml;
E: 0.2 ml heated lymph, incubated for 2 hours with adrenaline, 5 μg/ml;
F: 0.2 ml of a fresh Tyrode-adrenaline solution, 5 μg/ml.

Results.

The rate of adrenaline or noradrenaline inactivation in the various media — heated lymph, non-heated lymph and Tyrode solution — could be evaluated when the inhibitory action of the incubated samples on the rabbit's intestine was compared with equivalent amounts of fresh solutions of the two catechol amines (Fig. 1). Pure lymph, whether heated or not, had no or only insignificant effects on the intestine (A, B), even when given in much bigger amounts to the bath than was the case with the samples to which adrenaline or noradrenaline had been added. If, however, adrenaline in a concentration of *e. g.* 5 μg per ml had been added to normal lymph and incubated at 38° C for two hours the inhibitory effect induced on the intestine (D) was almost as marked as that obtained by an equivalent amount of fresh adrenaline solution (F). Evidently adrenaline is remarkably

stable in normal, non-heated lymph. Heating the lymph before adding adrenaline, other procedures being identical, produced a less prominent inhibitory response (E), indicating a greater loss of adrenaline in denatured lymph. The destruction of adrenaline was still more pronounced when it had been incubated with Tyrode solution for the same period (C) and often almost all adrenaline activity was under such circumstances lost after 2 to 3 hours.

No major differences from these results were observed if the amount of adrenaline added to the lymph samples was varied within rather wide limits or if noradrenaline was used instead of adrenaline. The elimination of adrenaline and noradrenaline when incubated in human lymph proceeded at essentially the same rate as when incubated in similarly treated lymph samples from the cat.

As regards another biogenic amine, histamine, it is well known that its inactivating enzyme, histaminase, is found in normal lymph in fairly high concentrations, which tremendously increases after adrenalectomy (CARLSTEN 1950). It was *a priori* not impossible that under such circumstances specific catechol amine destroying enzymes might also appear in the lymph in significant amounts. Lymph was therefore also collected from adrenalectomized cats, 1, 2, 4 and 24 hours after adrenalectomy had been performed. Even after these procedures, however, there was no evidence of any significant enzymic elimination of adrenaline and noradrenaline in the lymph, as the test results were essentially the same as those previously described. The small 'post-inhibitory excitation' of the intestine after the addition of normal lymph incubated with adrenaline (D), was a fairly regular phenomenon, which was not observed with similar samples of boiled lymph or Tyrode solution. The nature of this secondary effect was not revealed.

Discussion.

Already in 1905 when ELLIOTT first suggested the possibility that the sympathetic nervous system exerts its action by liberating a specific ergone at the nerve endings he also postulated that the substance might be destroyed at the site of release. Since then a great number of studies have dealt with the destruction of catechol amines in various tissues. The mechanism responsible for the elimination of adrenaline and noradrenaline in the organism is still widely discussed, however, and no general agree-

ment has so far been obtained (see BEYER 1946, BACQ 1949, BLASCHKO 1952, BURN 1952, SCHAYER and SMILEY 1953, STRÖMBLAD 1956).

Amine oxidase is by many authors thought to play an important rôle in this inactivation and it has even been suggested that it plays a part similar to that of choline esterase at cholinergic neuro-effector junctions. Its distribution and activity in different tissues have been frequently studied both *in vitro* (see *e. g.* BLASCHKO, RICHTER, SCHLOSSMANN 1936, 1937, BAIN et al. 1936, 1937) and by histochemical methods (KOELLE 1950, 1954). Fairly large amounts of amine oxidase have been demonstrated in *e. g.* the liver, while striated muscle seems to contain only little of this enzyme. Its actual concentration at the very site of release of the sympathetic transmitter is, however, very difficult to determine, and this localization, which may be characterized by a high concentration in a very small area, is of course of major interest. It should also be stressed that there might very well be a number of enzymes with a widespread distribution in the tissues, that are able to deal rapidly with physiological concentrations of the sympathetic ergones.

As regards *in vivo* studies of the elimination of adrenaline and noradrenaline it is difficult to judge the details of the destruction by such methods but they may give us an idea of the quantitative side of the elimination under conditions that are closer to a true physiological state. Relatively few such quantitative data are at present available, however, though it has been repeatedly observed that adrenaline and noradrenaline seem to disappear fairly rapidly from the tissues. CELANDER and MELLANDER (1955) recently described a simple *in vivo* method for determining the amounts of catechols eliminated in different tissues. These experiments indicate that the loss of adrenaline and noradrenaline during a single capillary passage is generally quite extensive.

From other experiments (see CELANDER 1954) it appears as if the sympathetic transmitter substance 'overflows' in significant amounts into the extracellular fluid, as represented by the blood plasma, only when definitely supraphysiological stimulation rates have been applied to the sympathetic nerves. Therefore it seems as if the transmitter substance is normally destroyed either at the very site of, or in close vicinity to the release. — In blood itself catechol amines seem to be very stable (BAIN and SUFFOLK

1936, BAIN, GAUNT and SUFFOLK 1936, 1937). They found that the approximate time for a 25 per cent inactivation of adrenaline in plasma was 180 minutes. In whole blood the picture was somewhat more complicated owing to the rapid uptake of adrenaline by the red corpuscles, but the major part of this uptake could be recovered by laking the blood cells. These data — with a bearing on the plasma as a specialized fraction of the extracellular fluid — are in good agreement with our results on lymph, which more intimately reflects the characteristics of the fluid creating the 'milieu interne' for the tissue cells. Normal lymph seems to be a preservative for catechol amines rather than a medium for accelerated destruction of these highly active substances. Therefore the conclusion seems to be that the physiological mechanisms responsible for destruction of released sympathetic ergones are strictly bound up with the tissue cells.

The fact that adrenaline and noradrenaline destruction is definitely delayed in normal, and to a lesser extent in denatured lymph as compared with *e. g.* Tyrode or Locke solution has probably to do with the proteins or protein-bound radicals. It might well be that adrenaline in some way combines reversibly with the protein molecules or radicals connected with these molecules and that the free oxidation of adrenaline under such circumstances is more or less inhibited. Denaturing the proteins by heating decreases but does not completely abolish the preserving capacity of lymph. These observations demand further study.

Summary.

1. The inactivation of adrenaline and noradrenaline in lymph collected from cats and, in one case, from a human being, was studied.

2. In lymph the sympathetic hormones are fairly stable and even after several hours at 38° C the loss is small. Denaturation of the lymph proteins accelerates the inactivation rate of the sympathetic hormones.

3. It is concluded that *in vivo* destruction of adrenaline and noradrenaline is strictly bound up with the tissue cells and that the extracellular fluid rather to some extent prevents catechol amine destruction, a mechanism probably connected with its protein content.

This study was made possible by a grant from the Faculty of Medicine, University of Gothenburg, Gothenburg.

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On the Intestinal Absorption of 2,2-Dimethylstearic Acid Fed as Free Acid or as Glyceride.

By

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Received 18 April 1956.

When fed to rats, 2,2-dimethylstearic acid is well absorbed and mainly incorporated into lymph glycerides. *In vitro* experiments have shown that glyceride ester bonds with this acid were almost resistant to the action of pancreatic lipase. Only traces of free acid were incorporated into glycerides by this enzyme *in vitro* (BERGSTRÖM, BORGSTRÖM, TRYDING and WESTÖÖ 1954). In one series of experiments we have now studied the absorption of glycerides containing incorporated [carboxy-^{14}C]2,2-dimethylstearic acid. In another series the intestinal contents have been studied during active fat absorption and thus the *in vitro* experiments with pancreatic lipase have been repeated *in vivo*.

Experimental.

2,2-dimethyl[1-^{14}C]stearic acid (BERGSTRÖM, BORGSTRÖM, TRYDING and WESTÖÖ 1954) with a specific activity of 25,000 counts/min/mg was used in this study. The labelled acid was fed as free acid or as glyceride to rats. In one series of experiments the lymph, faeces and urine were collected and studied (Group A). In another series the intestinal contents were analysed during the absorption phase, 1—2 hours after the administration (Group B).

Table I.

Recovery in lymph and urine after feeding 2,2-dimethylstearic acid as free acid (1, 2, 3) and as glyceride (4, 5) to rats.

Rat no.	Per cent of fed activity absorbed	Per cent of absorbed activity recovered in lymph lipids	Per cent of lymph activity recovered as			Per cent of absorbed activity recovered in urine
			glycerides	free acids[1]	phospho-lipids	
1	65.8	27.9	86.8	9.2	3.8	32.3
2	86.5	49.0	96.4		3.6	34.6
3	78.1	49.1	85.1	13.1	1.6	32.9
4	78.2	27.9	96.0		4.0	34.2
5	84.8	41.4	84.7	10.8	4.5	25.7

[1] When [1-^{14}C]palmitic acid was fed to rats with thoracic duct fistulas 6.0 and 8.3 per cent of the lymph activity was found in the free fatty acid fraction.

A. *Animals with a thoracic duct fistula.*

The animals used throughout this investigation were white adult male rats weighing about 250 g. The procedure used for cannulating the thoracic duct and the postoperative treatment has been described earlier by Bergström, Blomstrand and Borgström (1954). About 24 hours after the operation the rats were fed 0.75 ml of a 4 per cent solution of 2,2-dimethyl[1-^{14}C]stearic acid in olive oil by a stomach tube under a light ether anaesthesia. The lymph was collected for the next 24 hours.

The faeces collected for periods of 24 hours were saponified by refluxing for 12 hours with a solution of 30 % (w/v) KOH at 100° C. After acidification they were extracted with ether. The amount of activity absorbed was calculated from the difference between the amount of isotope administered and the activity recovered in the faeces.

The urine was diluted with a suitable amount of water and directly plated on aluminium planchets. Correction for self absorption was made as described by Bergström, Borgström, Tryding and Westöö (1954 a). For the measurements of radioactivity a thin end-window G-M tube was used with a background of 30 c.p.m. At least 1,000 counts were registered for each sample.

The lymph fat was extracted with 20 volumes of ethanol-ether (3 : 1) and after evaporation of this extract *in vacuo* the residue (2—3 ml) was extracted with light petroleum-chloroform (2 : 1). The total fat was separated into a neutral fat-fatty acid fraction and a phospholipid fraction on a column of silicic acid according to Borgström (1952 a). The neutral fat was separated from free fatty acids by chromatography on Amberlite IRA-400 (Borgström 1952 b).

The glyceride fraction was dissolved in a suitable amount of olive oil and used for administration to rats with lymph fistulas. In this

Table II.

The composition and isotope content in the lipids recovered from the intestinal washings after feeding 2,2-dimethylstearic acid as free acid (6, 7) and as glyceride (8, 9, 10) to rats.

Rat no.	Animals sacrificed hrs.	Total fat recovered mg	Composition of total fat		Activity recovered as per cent of total activity	
			% free fatty acid	% glycerides	glycerides	free acids
6	1 ½	48.6	38.0	62.0	2.6	97.4
7	2	178.8	36.0	64.0	2.0	98.0
8	1	212.0	24.6	75.4	98.6	1.4
9	1 ½	198.2	22.7	77.3	97.3	2.7
10	2	273.8	29.5	70.5	98.3	1.7

way each rat received about 200 mg of labelled lymph glycerides. The lymph, faeces and urine from the rats were collected and analysed.

B. *Non-cannulated rats.*

Glycerides containing 2,2-dimethylstearic acid were biosynthesized as described above and given in an olive oil solution to rats without a lymph fistula. Each rat was given 0.75 ml of an olive oil solution containing about 200 mg of glyceride. For comparison the labelled acid was also given as free acid dissolved in olive oil (the stock solution used in series A). The animals were killed 1—2 hours after the administration and the contents of the small intestine were washed out with saline into dilute hydrochloric acid. The total fat was extracted three times with two volumes of ether. The fat was then separated into glycerides and free fatty acids on a column of Amberlite IRA-400 (Table II).

Results.

The results from the lymph experiments are summarized in Table I. After feeding the labelled acid in free form, from 66 to 87 per cent of the fed activity was absorbed. The activity excreted in the urine was in the range of 30 per cent of the absorbed amount. The activity recovered in the lymph after feeding the free acid varied from 30 to 50 per cent of the absorbed activity. The main part of the lymph activity was found in the glyceride fraction. About 4 per cent of the activity was in the phospholipids and 9—13 per cent appeared as free acids. No significant difference between the two administration forms was found.

The experiments in group B are shown in Table II. The composition of the lipids recovered from the intestinal contents was 36—38 per cent of free acids and 62—64 per cent of glycerides when the free acid was fed dissolved in olive oil. These figures are in accordance with those found by BORGSTRÖM (1952 c). When the labelled glycerides were administered the values were 25 to 30 per cent of free acids and 71—75 per cent of glycerides. In the animals that had been given glycerides containing 2,2-dimethyl[1-^{14}C]stearic acid, however, only 1—3 per cent of the total radioactivity recovered in the intestinal contents was present as free fatty acids, while the remainder was still in the glyceride fraction. In the corresponding experiments with free labelled acid dissolved in olive oil almost all of the recovered activity was in the free acid form and only 1—2 per cent was incorporated into glycerides.

Discussion.

2,2-Dimethylstearic acid was well absorbed from the intestine when fed as free acid or as glyceride dissolved in olive oil. The extent of transport via the lymphatics was the same irrespective of the form in which the acid was fed. However, from the data in this investigation it is apparent that a somewhat lower absorption is obtained than for the ordinary straight chain fatty acids. A large amount (up to 35 per cent) of the absorbed activity was recovered in the urine of the rats with lymph fistulas, mainly as 2,2-dimethyladipic acid. These findings suggest that a significant amount of the acid might have been absorbed via the portal vessels. Whether the degradation of dimethylstearic acid to dimethyladipic acid had started already in the intestinal cells has not been determined.

In the present investigation it was found that when isolated lymph glycerides containing the labelled 2,2-dimethylstearic acid in ester linkage were subjected to the action of the intestinal enzymes only a small amount of the labelled fatty acids was split off. In accordance with this only traces of the activity were found incorporated into glycerides when the free acid was fed during the same conditions together with inactive olive oil. These results show that the labelled dimethylstearic acid fed as glyceride is absorbed mainly in glyceride form. These experiments thus confirm and extend the *in vitro* experiments with 2,2-dimethylstearic acid (BERGSTRÖM, BORGSTRÖM, TRYDING and WESTÖÖ 1954).

The major part of the lymph activity was found in the glycerides. A noticeable fact was that about 9—13 per cent of the lymph activity was present as free acids. Only about 4 per cent was incorporated into the phospholipids.

The same distribution pattern has been found earlier for 2,2-dimethylstearic acid (BERGSTRÖM et al. 1954 a) and for 2,2-dimethylnonadecanoic acid and 2,2,17,17-tetramethylstearic acid (TRYDING and WESTÖÖ, to be published).

The high incorporation of the acid in the lymph glycerides together with the results of the analysis of the intestinal contents support the suggestion made by BERGSTRÖM et al. (1954 a) that another mechanism than the ordinary pancreatic lipase system is concerned in the synthesis of new ester bonds in the intestinal mucosa.

The high content of free 2,2-dimethylstearic acid in the lymph as compared with the free fatty acid fraction of the lymph after feeding ordinary straight chain fatty acids (cf. BORGSTRÖM and TRYDING 1956) may be due to the stearic hindrance at the carboxyl group.

In earlier experimental works with labelled glycerol (REISER et al. 1952 and BERNHARD et al. 1952) the results have indicated that fatty acids may be absorbed in glyceride form. The results from the present investigation with glycerol ester bonds that are not split by pancreatic lipase definitely show that glycerides can be absorbed into the mucosa cells without previous total hydrolysis.

Summary.

1) 2,2-Dimethyl[1-^{14}C]stearic acid has been fed as free acid and as glyceride to rats with a lymph fistula and to noncannulated rats. The composition of the lymph lipids and the intestinal contents have been studied. No significant differences were found in the absorption and lymph composition when the acid was fed in the free form or incorporated into glycerides.

2) The enzymes in the intestinal lumina could not split or build up ester bonds with 2,2-dimethylstearic acid *in vivo*. With this type of glyceride ester bonds it has been definitely shown that glycerides can be absorbed into the mucosa cells without total hydrolysis.

This work has been supported by grants from the Medical Faculty of the University of Lund, from Knut och Alice Wallen-

bergs Stiftelse, and from the Swedish Natural Science Research Council.

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